A QUANTITATIVE METHOD FOR THE ASSAY OF VITAMIN D WITH CHICKENS

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TWO FIGURES

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The response of chickens, in comparison with the response of rats to a given antirachitic agent, is the principal means of investigating the multiple nature of vitamin D. This was the means used in distinguishing the vitamin D of irradiated ergosterol from that of cod liver oil, of irradiated cholesterol from irradiated ergosterol, and of tuna liver oil from cod liver oil (reviewed by Bills, '35). As increasingly fine distinctions come to be made, improved assay procedure is required.

Heretofore the best available methods for assaying an antirachitic agent with chickens consisted essentially in varying the dosage until a level was found, the effect of which more or less matched that of some standard product. Perfect matching was a matter of rare chance, hardly more significant statistically than the proximate matching obtained with many animals in gradations of the dosage. In the end, the potency of the test substance could be expressed only on a not-more-than or not-less-than basis, subject to an unknown and undeterminable probable error.

It is to be noted that this general type of vitamin assay procedure is still widely used. It is the method, with rats as the test animal, which was recently made official in U.S.P. XI ('35). Quantitative methods differ from it in providing means, such as a graph, whereby a) the response of the test

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group can be translated into unitage without the necessity of closely matching the standard response, and b) the probable error of the assay can be estimated. Such methods for the assay of vitamin D with rats have been the subject of detailed studies by several investigators, particularly Bills, Honeywell, Wirick and Nussmeier ('31), Bourdillon, Bruce, Fischmann and Webster ('31), Dyer ('31), Bourdillon and Bruce ('32) and Coward and Key ('33).

OUTLINE OF METHOD

A colony of single comb white Leghorn fowls is maintained on a normal ration containing, at all seasons, 200 international units of cod liver oil vitamin D per 100 gm. of feed.[1] Eggs from these hens are selected for hatching. All eggs of imperfect shell or abnormal shape and all which weigh less than 55 gm. the day they are laid, are discarded. Newly hatched chicks are removed to brooders which are heated and illuminated with incandescent lamps of ruby glass. No food is given the chicks before the third day of life, i.e., chicks hatched on Monday are first fed on Wednesday. From the third day of life and into the seventeenth day of life all chicks are fed our rickets-producing diet M, and given tap water to drink.

Diet M

Ground yellow maize	56
Wheat bran	10
Linseed oil meal	10
Wheat gluten	10
Skimmilk powder[2]	9
Calcium carbonate	2
Sodium chloride	1
Vegetable oil (vehicle for vitamin D supplement)	2

Analysis, protein 21.8, Ca 1.0, P 0.5. Ca: P = 2: 1,

The 2 weeks on diet M is a fore-period during which diseased chicks, weaklings and runts either die or are thrown out. The

[1] An appreciable amount of vitamin D is transmitted from hen to chick, according to Murphy, Hunter and Knandel ('36) and Bethke, Record, Wilder and Kick ('36). Ample vitamin D in the feed should minimize variations in the amount transmitted.

[2] Beware of low grade skimmilk powders made from neutralized milk. The lime used in such products is a possible source of serious error in rickets-producing rations, according to Scott, Hughes and Loy ('32).

remaining strong chicks almost without exception survive the subsequent 4 weeks test period. The fore-period serves also another purpose: It brings the chicks to a state approaching rickets, so that in the test period to follow they will develop severe rickets or become normal birds or show any in-between condition, depending upon the amount of vitamin D now added to the basal diet.

Our method is designed particularly for the assay of oils and powdered milks. When the substance to be assayed is an oil or an oil-soluble material, it is incorporated in the 2% of vegetable oil of diet M. We use maize oil or cottonseed oil, but probably any other edible, vitamin D-free vegetable oil could be substituted. When a vitamin D-bearing powdered milk is to be assayed, it is substituted for the skimmilk and oil of diet M. Thus with either class of test substance, the vitamin supplement is added without any essential change in the composition of the basal ration.

At the end of the fore-period, in the seventeenth day of life, the chicks are transferred to assay cages. Five chicks are placed in each cage, the floor dimensions of which are 50×55 cm. In such close quarters the birds are restricted as to exercise; this may or may not be a factor of importance. The cages are provided with screen bottoms. The room is dimly lighted and maintained at about 27°. Ten chickens constitute the standard test group. They are given diet M containing the supplement of vitamin D for 28 days. Both the diet and tap water are supplied ad libitum. The supplemented diet is made up fresh each week and kept cold when not is use.

During the twenty-eighth day of the assay period (forty-fifth day of life) the chickens are killed. One femur from each bird is immediately dissected out, and placed in boiling water for 1 minute. It is then scraped free of adherent tissue, care being taken to avoid removal of the still cartilaginous epiphyses (Bethke and Record, '34). Skillful bone cleaning is important. In rickety bones the cortex is so easily abraded that the scalpel used should be somewhat dull. The bones

are broken in three pieces, and extracted in a Soxhlet apparatus for 12 hours with alcohol and 12 hours with ether. The extracted bones are dried to constant weight at 100°, then ashed to whiteness in an electric muffle furnace, the temperature of which is gradually raised to 700° ± 20°.

TABLE 1

Interpretation of ash findings in assays with ten chickens

I	II	III		IV				V			
Ash	Probable error (b)	I.U. of vitamin D per 100 gm. of ration		Probable error (d)				Probable error (e)			
		C.l. oil	Irr. erg.	Cod liver oil		Irr. ergosterol		Cod liver oil		Irr. ergosterol	
%											
36.0	± 0.41	2.0	40	+ 22;	— 43	+ 41;	— 44	+ 23;	— 43	+ 41;	— 44
37.0	0.43	3.1	80	15;	15	22;	22	16;	16	22;	22
38.0	0.45	4.2	120	12;	12	15;	15	13;	13	16;	16
39.0	0.46	5.3	160	10;	10	12;	12	11;	11	13;	13
40.0	0.47	6.4	200	8;	8	9;	9	10;	10	11;	11
41.0	0.47	7.4	240	7;	7	10;	8	9;	9	11;	10
42.0	0.47	8.5	293	7;	6	11;	9	9;	8	13;	11
43.0	0.45	9.9	372	8;	7	13;	11	10;	9	15;	12
44.0	0.44	11.8	498	8;	7	16;	13	10;	9	17;	14
44.5	0.42	12.8	593	8;	7	19;	14	9;	9	19;	15
45.0	0.41	14.0	726	8;	7	21;	16	9;	9	22;	17
45.5	0.39	15.2	922	8;	7	25;	18	9;	9	26;	19
46.0	0.37	16.7	1240	8;	7	31;	20	10;	9	32;	21
46.2	0.37	17.4	1420	8;	7	35;	22	10;	9	35;	22
46.4	0.36	18.1	1660	8;	7	40;	23	10;	9	40;	24
46.6	0.35	18.8	1970	8;	7	47;	26	10;	9	47;	26
46.8	0.34	19.7	2410	8;	7	56;	28	10;	9	56;	29
47.0	0.33	20.6	3060	9;	7	70;	31	10;	9	71;	32
47.2	0.32	21.6	4100	9;	7	97;	36	11;	9	97;	36
47.4	0.31	22.8	5980	10;	8		42	12;	10		43
47.6	0.30	24.2	10000	12;	8		51	13;	10		52
47.8	0.29	25.9		15;	9			16;	11		
48.0	0.28	28.3		50;	11			51;	12		

The average percentage of ash in the fat-free, moisture-free femurs of the ten birds comprising the assay group is located in column I of table 1. The corresponding number of international units (I.U.) of vitamin D per 100 gm. of ration is read from column III. When the assay has been conducted without the use of a positive control group the probable error,

PE_e, expressed as per cent of the number of units, is read directly from column V. When a positive control group has been used (e.g., a standard reference oil or any other substance, in terms of which the test substance is being assayed), the probable error, PE_f, is calculated by substituting in formula 7 the value of PE_d taken from column IV. When the number of chickens is other than the regular number, ten, the calculations of PE_e or PE_f are computed from PE_b in column II, by substituting it in formula 8 and following through as described under 'Probable Error.'

EXPERIMENTAL

The single comb white Leghorn was chosen as the test bird because it is the variety of fowl most widely available and already the most commonly used in experimental studies.

The basal ration, diet M, was planned so that, except for the lack of vitamin D, it is a normal diet for young chicks. Constancy in its P content is favored by the multiplicity of P-bearing ingredients. Most of its Ca is supplied by the added $CaCO_3$.

The Ca: P ratio of the diet is 2: 1. Ratios between 2: 1 and 1: 1 are optimal for young chickens, yet, as is well known, chickens develop rickets, no matter how favorable the ratio, unless vitamin D is supplied. Thus an unbalance in Ca: P, essential in rickets-producing diets for rats, is not required for chicks. In fact, it may even be undesirable. Preliminary experiments with several hundred chicks were performed, in which Ca: P was varied from 4: 1 to 1: 4, and several levels of vitamin D were administered with each ratio. There was no advantage in the abnormal ratios, although they were indeed provocative of bone disorders, so much so that death within the assay period frequently ended the experiment.

The basis for interpreting the findings of an assay was developed from a study of response curves, or ideal series, shown in figure 1. These master curves were obtained by administering graded doses of vitamin D in the form of cod liver oil and of irradiated ergosterol. The cod liver oil was

rendered solely from the livers of the common Atlantic codfish, Gadus morrhua. It contained 77 I.U. ($\pm$ 5.6%) of vitamin D per gram and about 1300 I.U. of vitamin A per gram. These values are within the ordinary limits of variation for pure

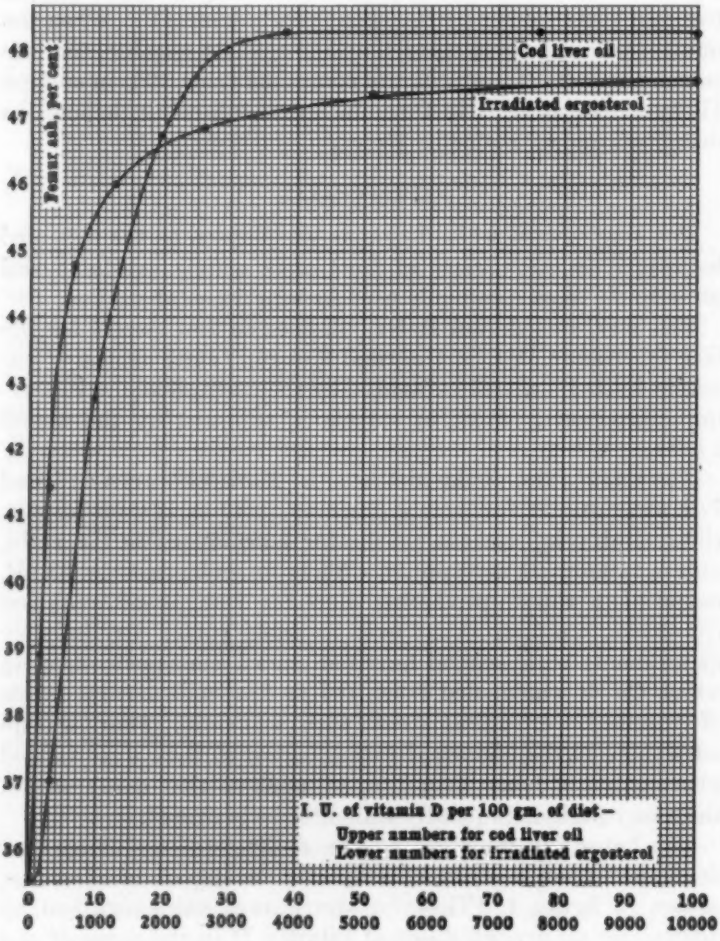

Fig. 1 Master curves of response.

cod liver oil, the average potency of which is 100 I.U. of vitamin D and 1000 I.U. of vitamin A per gram. They are somewhat lower than the values for many commercial specimens of 'cod liver oil,' the superior potency of which is due to the legally permitted admixture of liver oils from other species of the family Gadidae. The irradiated ergosterol was prepared as described by Bills, McDonald, BeMiller, Steel and Nussmeier ('31). It was dissolved in maize oil to give a stock solution containing 10,000 I.U. ($\pm$ 6.1%) of vitamin D per gram.

Dilutions of the cod liver oil and of the irradiated ergosterol stock solution were prepared in maize oil so that the desired unitage per 100 gm. of diet was supplied. Besides the zero level, 6 levels of the vitamin as cod liver oil and 7 as irradiated ergosterol, each indicated on the curves of figure 1, were triturated with the diet and administered to the chicks. The number of chicks used to establish each of the points determining the master curves was, of course, larger than the number for an ordinary assay. From twenty-five to fifty chicks (total, 519; average, 37) were used for each point, the larger groups being used at the ends and bends of the curves. The chickens were taken over the greater part of a year, and those of any given hatch were distributed over several dosage levels. Distribution as to sex was fortuitous.

On the curves of figure 1, the determining points were established by plotting the number of units administered per 100 gm. of diet as abscissas; the average percentages of femur ash corresponding to the unitage as ordinates. The general shape of the curves was not unexpected. Beginning at a threshold corresponding to the first definite betterment of the ricketic bone, and continuing halfway toward normal bone, the relation of units to ash is essentially linear. Thereafter, an ever increasing number of units of vitamin D are required to effect a degree of betterment in bone. Such, perhaps, is the course of most physiological responses in the direction of satiety.

Unless drawn on a very large scale, the curves in figure 1 cannot be read with sufficient accuracy for the interpretation of the ash findings in an assay. The curve readings in column III of table 1 were therefore not taken from the curves directly, but were calculated from equations of the curves which were determined for us by Mr. Delbert Deisinger.

A striking feature of the response curves for cod liver oil and irradiated ergosterol is their essentially different shapes. They cannot be made to coincide, by any magnification or contraction of abscissas. As drawn, they appear to intersect, but this appearance is an artifact due to the use of two series of abscissas for convenience in graphic portrayal. The artifact, however, draws attention to the great difference in the responses induced by these two forms of vitamin D. Where the curves 'intersect,' i.e., where approximately 46.5% of femur ash is obtained, the effectiveness of cod liver oil, rat unit for rat unit, is exactly 100 times as great as that of irradiated ergosterol for the chicken. Below this point, the difference in effectiveness gradually decreases, e.g., at 40.0% ash it is only thirty-one times. Above this point, the difference becomes great, e.g., at 47.3% ash it is 220 times. Thus we are able to explain the hitherto perplexing fact, discussed by Bethke, Record and Kennard ('33), that different investigators, comparing the efficacy of cod liver oil with that of irradiated ergosterol (or substances containing it), rat unit for rat unit on chickens, came to widely different conclusions. The two agents are simply not comparable in broad terms. Their relative effectiveness is determined by a number of factors, according to King, Hull and Hall ('33) and Bills, Massengale, McDonald and Wirick ('35). It now appears that the most important factor is the dosage at which the vitamin is given.

Under the conditions of our experiments, it is reasonable to regard a femur ash of between 46 and 47% as normal (compare fig. 1). Thus qualified, the effectiveness of the vitamin D of cod liver oil is 100 times that of irradiated ergosterol, rat unit for rat unit on chickens. This is the value originally

established by Massengale and Nussmeier ('30) under somewhat different experimental conditions. The amount of cod liver oil vitamin D required to give 'normal' bone with the 'normal' diet used is 18 I.U. per 100 gm. of ration.

PROBABLE ERROR

Estimation of the probable error in any given assay by means of table 1 is manifestly simple, but the computations by which table 1 was developed involve a chain of considerations. Several ways of expressing error were considered, but the method chosen as the most useful was that which expresses it as percentage of the number of units found by the assay.

One determinant of the probable error is the deviation to which the femur ash percentage of the ten chicks comprising the assay group is subject. Since the data from any individual group without reference to other groups are not very significant statistically, it seemed rational, as well as exceedingly convenient, to determine the average deviation, once and for all, by the analysis of many groups. This was done with the data from 160 groups of ten chicks each, including most of the chicks used in the ideal series. The average deviation in femur ash percentage was separately reckoned for each of the 160 groups. Still separately, these average deviations were converted into the probable errors of the groups by multiplying by 0.8453 (Peter's factor) and dividing by the square root of one less than the number of chickens in each group,

$$\text{Formula 1:} \qquad PE_a = \frac{0.8453 \times AD}{\sqrt{10-1}} = \frac{0.8453 \times AD}{3}$$

Thus we obtained 160 evaluations of PE_a, the probable error in ash percentage, which were to be expressed later as probable error in number of units, PE_c, and in per cent of units, PE_d. Separate computations showed no significant differences in PE_a when cod liver oil, other fish oils, or irradiated ergosterol supplied the calcifying agent.

At this point, a second determinant of error was found in the fact that the deviations in ash varied with the amount of ash. Bones of nearly normal ash content varied less than the

softer bones, perhaps as the result of their proximity to the limit of calcification or perhaps because they were less susceptible to mutilation in the hands of the assayist. The most variable bones were those partially calcified. Whatever the reasons for the variations, the fact precluded the averaging of the 160 values of PE_a and necessitated a graphic interpretation of them. The probable errors in ash were located on coordinate paper as shown in figure 2. Here the trend is clearly discernible despite the irregularities in the ordinates, which, as a matter of fact, are exaggerated ten times with

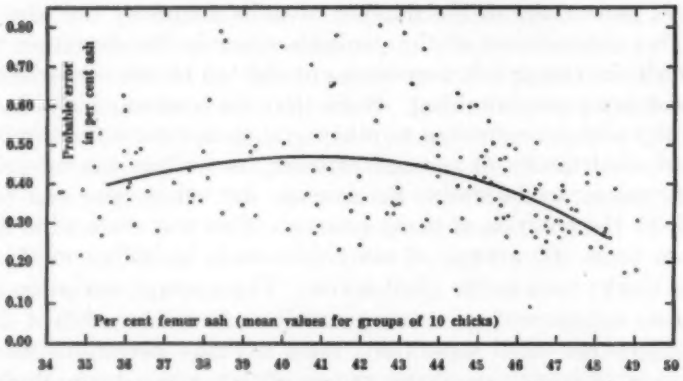

Fig. 2 Error in ash as a function of the amount of ash. (The vertical scatter of points is exaggerated ten times, for convenience in illustration).

respect to the abscissas as a convenience in making the drawing. No points were thrown out, however extreme the error which they represented. The points were arithmetically consolidated into ten clusters of sixteen points each, taken in order of ash percentage. From the ten new points representing the cluster centers, the curve in figure 2 was constructed. The curve broadens our knowledge of the errors, previously restricted to 160 instances, so that we can now state the probable error in ash which is associated with any degree of calcification in the typical assay with ten chickens. In distinction from PE_a, we designate the generalized curve readings as PE_b (column II in table 1).

The third determinant of error is the varying relation between ash produced and unitage required to produce it. This is represented in the slopes of the curves in figure 1, from which one notes that a given amount of difference in ash affects the number of units less where the curves are steep than where they have flattened out. Error in ash was translated into error in units, PE_c, in accordance with the equations,

$$\text{Formula 2:} \quad PE_{c,\ plus} = U' - U, \text{ and}$$
$$\text{Formula 3:} \quad PE_{c,\ minus} = U - U'', \text{ where}$$

PE_c is the probable error, plus or minus, to which the number of I.U. of vitamin D per 100 gm. of ration, corresponding to the found percentage of femur ash in ten chicks, is subject; U is the number of I.U. of vitamin D required in 100 gm. of ration to give the found percentage of femur ash; U′ is the number of I.U. of vitamin D required in 100 gm. of ration to give a percentage of femur ash equal to the found percentage plus its probable error, PE_b; and, U″ is the number of I.U. of vitamin D required in 100 gm. of ration to give a percentage of femur ash equal to the found percentage minus its probable error, PE_b.

The values for PE_b were read from an enlargement of the curve in figure 2, and the values for U, U′ and U″ were calculated from the equations of the curves of figure 1.

The fourth determinant of error, when error is re-expressed percentagewise, is the number of units per 100 gm. of ration. Since U, in the necessary calculations, is the divisor, it is apparent that error in per cent, designated PE_d, is greatest when U is smallest. The equations are obvious:

$$\text{Formula 4:} \quad PE_{d,\ plus} = \frac{PE_{c,\ plus} \times 100}{U} \text{ and}$$

$$\text{Formula 5:} \quad PE_{d,\ minus} = \frac{PE_{c,\ minus} \times 100}{U}$$

In table 1 the values for PE_d represent the error which is inherent in the method of comparing a group of ten chicks with an errorless ideal series, no controls being used. But a fifth determinant of error enters here, in the fact that the cod liver oil and the irradiated ergosterol solution with which the master curves were established, were themselves subject

to an assay error, PE_s. They had been standardized with rats against the international reference solution of irradiated ergosterol issued by the Health Organisation of the League of Nations ('31). The procedure was that described in our critique of the line test (Bills et al., '31), with certain minor improvements. The probable error of the standard, PE_s, was $\pm$ 5.6% with the cod liver oil, and $\pm$ 6.1% with the irradiated ergosterol. Taking this into consideration, we finally arrive at the value for the probable error, PE_e, expressed in per cent of the number of units found by an assay with ten chickens, without any controls other than those represented in the master curves,

Formula 6: $$PE_e = \sqrt{PE_d^2 + PE_s^2}$$

Values of PE_e are tabulated in column V of table 1.

It is sometimes desired to assay one vitamin D source in terms of another, rather than in terms of the master curves. For instance, one may wish to assay a sample of cod liver oil in terms of an official reference oil.[3] In such assays two groups of ten chickens each must be used, one for each of the substances. The error, PE_s, is eliminated, but another error is introduced by the additional assay group. The probable error of the end result, PE_f, is found by a modification of formula 6, namely,

Formula 7: $$PE_f = \sqrt{PE_{d1}^2 + PE_{d2}^2}$$

where PE_{d1} and PE_{d2} are the PE_d values of the two groups of chicks. Values of PE_f are not given in the tables, since they must be reckoned separately for each problem that arises.

To illustrate the use of formula 7, suppose that two cod liver oils, X and Y, were assayed with ten chickens each. Oil X showed 160 I.U., and oil Y 80 I.U. of vitamin D per gram.

[3] Although the U.S.P. XI ('35) unit and the international unit are by definition identical, it does not follow that the declared potency of the U.S.P. reference cod liver oil is free of error in terms of the international standard. Our experience has been that the current (first) issue of the U.S.P. reference oil, which nominally contains 95 U.S.P. units of vitamin D per gram, actually shows but 80 $\pm$ 2 I.U. of vitamin D per gram when assayed most carefully with rats against the international standard. It is emphasized that our master curves were based upon the international standard, not upon the reference oil.

Each value, of course, was subject to error. For most purposes the computations would end here, but if it were desired to express X in terms of Y, one would divide 160 by 80, finding that X is two times as potent as Y. To determine the probable error of this quotient, one would make a special computation from the PE_d values in column IV instead of taking the PE_e values in column V. Let us say that the PE_d values of the two assays were x and y, respectively. Substituting in formula 7,

$$PE_r = \sqrt{x^2 + y^2}$$

which is the probable error of oil X in terms of oil Y. This value is usually somewhat greater than the larger of the PE_e values of the two assays. Even when oil Y is an official standard, such as the U.S.P. reference cod liver oil (which by definition contains a certain number of U.S.P. units[3]), the procedure remains the same for the computation of error. After the quotient of X over Y has been determined, one multiplies this quotient by the officially defined potency of Y, to express the potency of X in terms of the units used in the official definition of Y.

The sex error. In the line test with rachitic rats it has been reported that males deposit slightly more calcium than females in response to semi-curative doses of vitamin D (Bills et al., '31; Bourdillon et al., '31; and Coward and Key, '33). The difference is so small, and so interwoven with other variables that investigators agree it is of no practical significance. In studies with normal chickens it has been found that male tibias contain slightly less ash than female tibias. From the work of Holmes, Pigott and Moore ('32) and Schroeder ('33) it appears that the ratio of tibia ash percentage in males to that in females varies with the age of the chicks as follows:

21 days — M: F = 101.1 : 100 (Holmes et al., '32)
42 days — M: F = 97.7 : 100 (Holmes et al., '32)
63 days — M: F = 96.8 : 100 (Holmes et al., '32)
70 days — M: F = 96.4 : 100 (Schroeder, '33)

If a similar relation holds for femur ash, one can, by interpolation, estimate that for 45-day chicks the M: F ash ratio is about 97.5: 100. On this basis, an assay of cod liver oil con-

ducted with cockerels only would figure out 8% fewer units than one with both sexes, and an assay with pullets only would figure out 7% more units than one with both sexes. (It is assumed that the chicks with which the master curves were constructed had a rex ratio of 1:1). Similarly, if the substance assayed were irradiated ergosterol, the differences would amount to 9% and 12% for cockerels and pullets, respectively. These calculations are based on a found femur ash of 41.4%, wherewith the associated probable error is approximately minimal.

It should be borne in mind that all calculations of the probable error in our method include the error due to sex. It remains now to be determined whether or not reduction of the total probable error by correction for sex error is feasible. It is, of course, impossible for the average worker to ascertain the sex of chickens at the beginning of an assay, so that the correction, if made at all, would have to be made at the end.

Assuming that the sex ratio of chickens is unity, one learns by the factorial equation,

$$_nC_r = \frac{n!}{r!\,(n-r)!}$$

that the chances of any assembly of ten chicks being either all cockerels or all pullets are only two in 1024. But the chances are 912 in 1024 (about 89%) that the sex ratio will be one or another of the following, 7:3, 6:4, 5:5, 4:6 or 3:7. Here the sex error is inconsequential. There remain 110 chances in 1024 (about 11%) that a ratio of 2:8, 8:2, 1:9 or 9:1 will obtain, in which the sex error is small, but appreciable.

The importance of sex, small as it thus appears, is even smaller, for it has been shown by Lachat ('34) and Lachat and Halvorson ('36 a, b) that in rachitic chicks, unlike chicks that have received adequate or nearly adequate amounts of vitamin D, the bone ash difference associated with sex is insignificant. It therefore would seem that in our procedure, correction for sex error is not worth while.

Use of controls. Were it possible to have an assay technic flawless in every detail, controls would merely confirm the master curves upon which the technic was based. Under

practical conditions, negative controls have little value, but positive controls are useful. The latter serve to assure that gross errors, such as incorrect compounding of basal diet, have not entered the work. They also compensate for periodic errors, such as the seasonal variation in bone ash which Lachat ('34) and Lachat and Halvorson ('36 a, b) have observed in rachitic chickens. To some extent, however, the error due to seasonal variation is already included in the calculations of probable error in our method.

When the positive controls are run merely to assure that the response of the test birds is not materially out of line with the master curves, i.e., when error is to be estimated as PE_e of column V in table 1, it is well to remember that error is as likely to occur in the control group as in the test group, and that deviations in the control group average from the master curves of less than three times the probable error need not be considered significant unless they are consistently repeated. When the positive controls are run as a standard reference oil, in terms of which the potency of the test substance is to be calculated, i.e., when error is to be computed as PE_t via PE_d of column IV in table 1, it should be recognized that control group error is not thereby eliminated, but is merely hidden.

Use of a number of chickens other than ten. All data on error in table 1 are based on the use of ten chickens in each assay group. It is possible, however, to adjust the calculation of error to numbers of chickens other than ten. In so far as error is determined by the number of birds, it varies inversely with the square root of one less than the number, but since there are other determinants of error than number of birds, one cannot apply this inverse relationship to the final calculations of PE_e or PE_t. One must introduce the correction in PE_b, and thence carry it through each successive formula to the final computations. It is for this purpose that values of PE_b are given in column II of table 1. To obtain the value of PE_b adjusted for n birds, one solves—

$$\text{Formula 8:} \quad PE_{b_n} = \frac{\sqrt{10-1} \times PE_b}{\sqrt{n-1}} = \frac{3 \times PE_b}{\sqrt{n-1}}$$

The corrected values thus obtained are not wholly valid, especially for small groups, because certain errors, such as the error due to sex, increase disproportionately when the number of animals is substantially less than ten.

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SUMMARY

1. A quantitative method is described for the assay of vitamin D with chickens. It is based on the determination of femur ash, and the interpretation of the ash percentage by means of either of two response curves, one for cod liver oil and the other for irradiated ergosterol.

2. The method has been analyzed in regard to probable error, and means provided for the easy estimation of error, with any given number of chickens, in both direct assays and in assays of one substance in terms of another. Under the most favorable conditions the probable error of an assay of irradiated ergosterol with ten chickens is as little as 9% of the unitage found. A slightly smaller error is experienced in assays of cod liver oil.

3. With the diet used, which had Ca : P = 2 : 1 (optimal for young chicks), good calcification resulted from the addition of 18 I.U. of vitamin D, as cod liver oil, per 100 gm. of ration.

4. Explanation of conflicting reports on the relative effectiveness, rat unit for rat unit, of cod liver oil and irradiated ergosterol for chickens, was found in the fact that these sources are not broadly comparable. Their relative efficacy fluctuates with the degree of calcification, the difference being small at low calcifications, 100 times at 'normal' calcification, and very great in the production of extra-hard bone.

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SUSCEPTIBILITY OF DIFFERENT STRAINS OF RATS TO NUTRITIONAL CATARACT[1]

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ONE FIGURE

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In earlier papers (Mitchell, '35; Mitchell and Dodge, '35) the author reported the occurrence of cataract in white rats fed on lactose and galactose. The incidence of mature bilateral cataract in this first series of experiments in which 70, 50 and 30% lactose rations were fed was 69, 27 and 0%, respectively. The incidence was 100% on the 35% galactose ration. All rats in these experiments were from the Battle Creek colony which originated 12 years previously from a cross between Wistar and Yale strains and were between 24 and 30 days of age when started on the experimental rations.

Yudkin and Arnold ('35) using rats from the Yale colony obtained less than a 50% incidence of cataract on the 70% lactose ration. A limited number of rats from the University of Wisconsin colony fed on the 70% lactose ration developed mature cataract at a slower rate than animals from our own colony. A personal communication from Michigan State College informed us that in their colony a few cataracts developed when high levels of lactose were fed. The rations were not exactly like those in our work nor was the exact incidence of cataract recorded. Such casual observations,

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however, suggested a possible variation in susceptibility to cataract among different strains of rats.

When the cataract research project was reestablished in a new institution rats from the local colony (M.S.C.) were used in some preliminary experiments. They proved exceedingly resistant to lens changes of the more severe type. Rats from a pure Wistar strain (W.) were next used on the 70% lactose ration and cataracts again failed to develop in the usual time. Finally rats from the Battle Creek colony (B.C.) were again tested and found to be equally if not slightly more susceptible to cataract than in our previous series of experiments with the same strain. This striking difference in breeds which retarded progress in our main research program nevertheless prompted us to make further observations on the response of different strains to the same rations. More animals from each of the albino strains mentioned were fed the two cataract producing rations. Later a group of 'pied' rats (J.H.) from the biochemistry department of the Johns Hopkins University were fed the galactose diet.

EXPERIMENTAL

Procedure. Growth, food intake, abnormal intestinal conditions, early lens changes and incidence of mature cataract were recorded regularly for all animals. Table 1 summarizes the significant findings on the three strains of rats fed on the 70% lactose ration and the four strains fed on the 35% galactose ration. The number of rats from the B.C. strain was naturally much greater than from any of the others since it proved expedient to use rats from this colony for all subsequent observations on cataract. The possibility of a sex difference in susceptibility to cataract was also considered, for which reason the data on males and females were recorded separately.

Susceptibility to cataract. The incidence of cataract in the various breeds and the time required for development of complete opacities is graphically told in figure 1. On the 70% lactose ration the high incidence of mature cataract in the

ninety-eight rats of the B.C. strain is the most significant. Advanced lens changes appeared in all of the animals and mature cataract was visible to the naked eye in 82 and 89% respectively of the males and females. It is obvious that the

TABLE 1

Response of different strains of rats to lactose, galactose and starch rations

RATION	STRAIN	NUMBER OF RATS	RATE OF GROWTH	INTESTINAL CONDITION	TIME IN DAYS FOR DEVELOPMENT OF MATURE CATARACT			INCIDENCE OF CATARACT PER CENT
					Min.	Max.	Average	
70% lactose	B.C.♂	52	Delayed	Severe	14	80	44	82
	B.C.♀	46	Delayed	Diarrhea	18	104	45	89
	M.S.C.	10	Delayed	Diarrhea	49	63	56	10
	W.	11	Delayed	Distention and diarrhea	97	97	97	9
35% galactose	B.C.♂	34	Normal	Normal	11	26	16	100
	B.C.♀	42	Normal	Normal	12	28	17	100
	M.S.C.	6	Normal	Normal	21	44	32	100
	W.	10	Normal	Normal	21	49	29	60
	J.H.	15	Normal	Normal	16	45	23	83
70% starch	B.C.	55	Normal	Normal	..	..	..	0
	M.S.C.	4	Normal	Normal	..	..	..	0
	W.	4	Normal	Normal	..	..	..	0
	J.H.	4	Normal	Normal	..	..	..	0

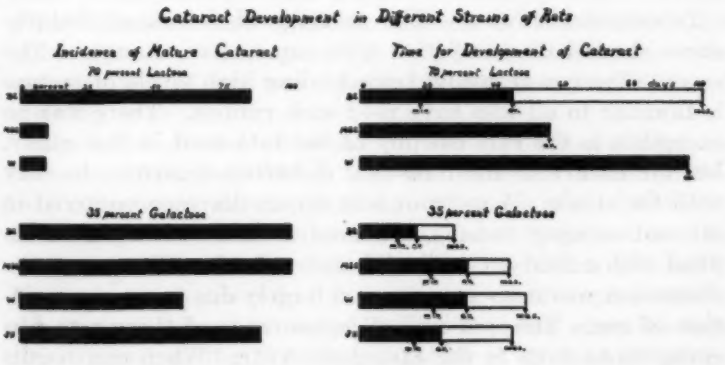
Cataract Development in Different Strains of Rats
Incidence of Mature Cataract
Time for Development of Cataract
70 percent Lactose
35 percent Galactose

Figure 1

difference between the sexes is insignificant. The longer time recorded for cataract development in the other strains becomes more striking when one notes that only two eyes out of twenty in the M.S.C. strain and two out of twenty-two in the W. strain ever showed complete opacities, although all of the animals from these strains remained on the 70% lactose ration well beyond the time when cataract might be expected to develop.

On the 35% galactose ration the incidence of mature cataract is high in all four strains, 100% in the B.C. and M.S.C. rats. The time required for the development of cataract in the different breeds is particularly interesting. The time is shortest and surprisingly constant for the B.C. rats, the others showing a slower development on the average and a much greater variation in time within a given litter.

Growth. There was no apparent difference between strains as regards rate of growth on the two rations. All showed delayed growth on the 70% lactose diet but tended to resume a better rate of growth after a few weeks when a tolerance for this sugar seemed to have developed. All strains grew at an approximately normal rate on the 35% galactose diet for the few weeks during which they remained on this ration. The galactose feeding was usually discontinued as soon as cataract developed, that is within 3 or 4 weeks.

Intestinal condition. The severity of intestinal disturbances showed no correlation with cataractous changes. The severe diarrhea resulting from feeding high levels of lactose is familiar to all who have used such rations. There was no exception to the rule for any of the rats used in this study, but the nature of the intestinal disturbance seemed to vary with the strain. A more or less severe diarrhea occurred in all and autopsy usually disclosed a much enlarged cecum filled with a fluid or semi-solid mass. In the Wistar rats the distension was more extreme and largely due to an accumulation of gas. The 'pot bellied' appearance of these rats was conspicuous even to the casual observer. When one recalls that this same strain was peculiarly resistant to lens injury

from this lactose diet, the question naturally arises as to how much of the sugar was actually absorbed into the blood stream. It is regretted that systematic blood sugar studies could not have been made on all animals but it is a promising field for further investigation. In a limited series of determinations made under standard conditions the total blood sugar levels were high, chiefly due to the non-fermentable fraction. Breed difference in this respect has not been investigated. Since completing this study the author has discovered a pertinent comment in a recent paper by Donhoffer ('35) who states that each breed of rat is a law unto itself as regards the rate of absorption of various sugars. Therein may lie the secret of the breed difference in susceptibility to cataract.

There was no apparent diarrhea or other intestinal disturbance in any of the rats on the 35% galactose ration regardless of the strain. The potent cataract producing action of galactose has previously been attributed to the inability of the rat to utilize this sugar as rapidly as it is absorbed from the gut (Mitchell, '35). The suggestion made by Dodge ('35) that there may be a correlation between the severity of intestinal abnormalities observed on the lactose rations and the incidence of cataract is quite definitely contradicted by these findings. The most rapid lens changes occur in rats where the intestinal condition appears to be normal. His conclusions are based upon a far too limited number of data and the use of but one basal diet for the production of cataract.

Age when started. The age at which rats are started on the experimental rations is also an important factor in the rate of cataract development as well as in total incidence. Although no difference in strain was noted in this respect all comparisons should be made with the age factor as nearly constant as possible. In our earlier series of experiments a slower development of lens changes was noted when the rats were started on the 70% lactose ration at 60 days of age in contrast to the usual procedure in which the age range was

from 25 to 30 days. In a later series of experiments, rats started at 120 days of age failed completely to develop mature cataract although they showed some early lens changes when observed with the ophthalmoscope. Yudkin and Arnold ('35) noted delayed cataract development in older rats on galactose rations. Young rats do not tolerate an abrupt change from a stock ration to the 70% lactose ration, which necessitates a gradual increase to this level over a period of 3 or 4 days. The 35% galactose ration is well tolerated by rats 25 days old or even younger.

CONCLUSIONS

It is quite possible that breed difference in rats used may account for the apparent discrepancy in observations from various laboratories. Bourne and Pyke ('35) reported a lower incidence of cataract due to vitamin G (B_2) deficiency than did Langston and Day ('33) and Day, Langston and O'Brien ('31). Although this is another type of nutritional cataract than the one reported in this paper it is possible that the difference in breed of rats and a possible difference in the time of starting on the experimental ration has a bearing upon the results obtained. These variations in the incidence of cataract in different strains and at different ages emphasize the necessity for a consistent experimental procedure, a relatively large number of observations, and a consideration of the susceptibility to cataract of the strain of rats used.

SUMMARY

1. Rats from different colonies fed the lactose or galactose cataract producing rations showed variable susceptibility to cataract.

2. The B.C. strain had the highest incidence and the mature cataracts developed in the shortest time of any of the three breeds tested on the 70% lactose ration.

3. The M.S.C. and W. strains showed advanced lens changes but complete opacities developed in only 10 and 9%, respectively, of the rats tested.

4. On the 35% galactose rations the incidence of mature bilateral cataract was 100% in B.C. and M.S.C. strains and slightly less in W. and J.H. strains. The time for cataract to develop was shortest in the B.C. strain.

5. Severity of intestinal disturbances did not correlate with cataractous changes in the lens.

6. Growth was normal on the galactose but slightly retarded on the lactose ration, probably due to the persistent diarrhea in the young rats.

7. Cataract development was more rapid in young rats than in older ones. The age at which animals are started on experimental rations must be kept constant if results are to be consistent.

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THE DISTRIBUTION OF VITAMIN B_4 IN SOME PLANT AND ANIMAL PRODUCTS

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Studies on vitamin B_4 hitherto reported have been concerned for the most part with concentration of this factor, and very little information as to its distribution has been accumulated. Reader ('29, '30) successfully used yeast as a starting material for the concentration of vitamin B_4, and a previous report from this laboratory (Keenan et al., '33) described the preparation of concentrates from pork liver, blue grass and alfalfa. Chicks were used as the experimental animals in the assay of these concentrates, and evidence was presented to show that the chick paralysis observed here was a manifestation of the same deficiency as the paralysis in rats described by Reader. Reader ('29, '30) presented two methods of assay, one involving the use of young growing rats and the other depending upon the use of adult rats. However, growing chicks are much more susceptible to vitamin B_4 deficiency than are rats, and hence are of greater value for assay of this factor. Recent improvements in the vitamin B_4-low ration for chicks, as described in a previous paper (Kline et al., '36), suggested a study of vitamin B_4 distribution.

EXPERIMENTAL

Day-old White Leghorn chicks were obtained from the department of poultry husbandry for these experiments and housed in 14 inch by 16 inch by 14 inch cages equipped with

[1] Published with the permission of the director of the Agricultural Experiment Station.

wire screen floors. The basal ration used in these assays was ration 452, which has the following composition:

Dextrin	64
Reprecipitated casein	18
Salts l	5
Brewer's yeast	2
Autoclaved liver residue	2
Liver extract	2
Water extracted lung	5
Cod liver oil	2

TABLE 1

Vitamin B_4 content of seeds and seed products

SUPPLEMENT TO RATION 452	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS	AVERAGE WEIGHT 6 WEEKS	ONSET OF PARALYSIS	NUMBER WITH DEFICIENCY	PER CENT PARALYSIS	DEATHS PRIOR TO 6 WEEKS
				weeks			
None	29	77	...	3	29	100	27
20% Crisco	3	80	134	..	0	0	0
32% Wisconsin 1933 yellow corn	3	106	270	3	2	66	0
32% Wisconsin 1934 yellow corn	3	82	230	3	2	66	0
32% Wisconsin 1935 yellow corn	3	90	287	3	2	66	0
40% Wisconsin 1935 yellow corn	3	148	423	..	0	0	0
24% Texas 1935 yellow corn	4	103	295	2	1	25	1
32% Texas 1935 yellow corn	4	103	315	2	2	50	2
40% Texas 1935 yellow corn	3	152	382	..	0	0	0
24% white corn	4	95	180	3	1	25	1
32% white corn	3	108	285	..	0	0	0
16% wheat	3	90	212	2	3	100	0
24% wheat	3	87	177	4	3	100	0
32% wheat	6	115	320	5	4	66	0
40% wheat	3	135	323	..	0	0	0
40% autoclaved wheat	3	115	320	3	2	66	1
24% hulled oats	4	115	358	3–5	3	75	0
32% hulled oats	4	91	282	..	0	0	0
10% wheat germ	4	75	243	6	1	25	1
15% wheat germ	12	137	350	..	0	0	0
20% wheat germ	3	152	450	..	0	0	0
5% peanuts	6	83	...	3	6	100	6
10% peanuts	6	108	271	4	6	100	0
15% peanuts	20	130	355	..	0	0	0
10% NaCN dried grass	6	132	285	..	0	0	0
15% defatted wheat germ	4	136	...	3	4	100	4
20% defatted wheat germ	3	143	325	..	0	0	0

A detailed description of the constituents of this ration is given in a previous paper (Kline et al., '36). The various levels of the materials to be assayed were substituted in this ration for equal amounts of dextrin.

TABLE 2

Vitamin B_4 assay of animal products

SUPPLEMENT TO RATION 452	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS	AVERAGE WEIGHT 6 WEEKS	ONSET OF PARALYSIS	NUMBER WITH DEFICIENCY	PER CENT PARALYSIS	DEATHS PRIOR TO 6 WEEKS
				weeks			
12% dried egg yolk	3	130	297	3	1	33	1
10% vacuum dried pork liver	4	66	238	..	0	0	0
18% vacuum dried pork liver	4	108	298	4	2	50	1
24% vacuum dried pork liver	3	175	452	..	0	0	0
15% dried pork brain	3	130	305	..	0	0	0
15% NaCN dried pork brain	3	147	360	..	0	0	0
10% NaCN dried pork brain	4	97	242	4	1	25	1
15% skimmilk powder	3	82	...	3	3	100	3
10% pork kidney (dried)	3	102	248	6	2	66	0
15% pork kidney (dried)	3	130	340	..	0	0	0
15% spinal cord	3	75	...	4	1	33	3

TABLE 3

Vitamin B_4 potency of plant and animal products

	Minimum protective level, per cent		*Minimum protective level, per cent*
NaCN dried grass	< 10	White corn	32
Peanuts	15	Hulled oats	32
Wheat germ	15	Wheat	40
Pork brain	15	Yellow corn	40
Pork kidney	15	Egg yolk	> 12
Crisco	< 20	Skimmilk powder	> 15
Defatted wheat germ	20	Spinal cord	> 15
Pork liver	24		

Tables 1 and 2 give the results of assays of plant and animal products, respectively. The grains used were obtained locally except for the sample of Texas yellow corn which was furnished by Dr. A. R. Kemmerer of the Texas Agricultural Experiment Station. The wheat germ and defatted wheat germ were commercial products sold for human consumption under the trade

names 'Embo' and 'Vio Bin,' respectively. The peanuts were unroasted, but dried at 50°C. The materials designated 'NaCN dried' were dried at room temperature after first adding sufficient NaCN solution to bring the concentration to M/1000. The vacuum dried pork liver and spinal cord were obtained from Dr. David Klein of the Wilson Laboratories, Chicago, and the other animal products were obtained from a local packing plant and dried at 50°C. unless otherwise indicated.

Table 3 gives the minimum protective levels of the materials assayed.

DISCUSSION

The data here presented show the grains to be relatively poor sources of vitamin B_4. The superiority of white corn and hulled oats to yellow corn and wheat is of interest, as is also the superiority of wheat germ to whole wheat. If the minimum protective level be taken as the criterion, it would appear that wheat germ was almost three times as potent as whole wheat. The various samples of yellow corn were assayed because it was felt that differences in such conditions as moisture, temperature, etc., during the growing season might have some effect on the vitamin B_4 content. The data do not show any appreciable difference in incidence of paralysis among the groups on the different types of yellow corn, but better growth was obtained in the groups receiving the Texas corn than in those receiving Wisconsin corn. However, only very limited significance can be attached to growth rates in experiments of this kind since it is not definitely known that the basal ration is complete in all factors other than vitamin B_4.

Peanuts appeared to be about twice as potent in vitamin B_4 as the best of the cereals, and among all the materials tested were excelled only by the grass. Some difficulty was experienced in drying the grass in such a way as not to decrease its B_4 content; hence the practice of adding NaCN to inhibit oxidative enzymes during the drying process was adopted. This method was also used with other materials, as indicated in table 2. The figures given here for brain tissue do not

indicate any advantage for the cyanide treatment, but it was found that only by this method could reproducible results be obtained and that brain tissue dried in the ordinary manner was very variable as to B_4 content. The results obtained with the sample of spinal cord seem to indicate that this tissue does not share the relatively high potency of brain. There was some variation in the vitamin B_4 potency of the vacuum dried liver. It was reported in an earlier paper (Keenan et al., '33) that 18% of this liver was sufficient, but in these experiments 24% was required to give protection from paralysis. The fact that the basal ration used in the earlier work probably was not as low in vitamin B_4 as the basal ration used in these experiments needs to be taken into account. As shown in the table, pork kidney ranked as equal to pork brain, peanuts and wheat germ. Like the other animal tissues, it is subject to the disadvantage that it requires careful drying to avoid destruction of the vitamin. Since this disadvantage does not apply to peanuts, they would appear to be a good source material to use in further work.

SUMMARY

1. A number of food materials have been assayed for vitamin B_4 potency using chicks as the experimental animals.

2. Dried grass, peanuts, wheat germ, pork brain and pork kidney were found to be good sources of vitamin B_4.

3. The grains tested were found to be relatively poor sources, but white corn and hulled oats were definitely superior to wheat and yellow corn.

We are indebted to Dr. David Klein of Wilson Laboratories, to Dr. A. R. Kemmerer of the Texas Agricultural Experiment Station, to General Mills, Inc., and to the Vio Bin Corporation, Chicago, for supplying materials for these assays.

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VITAMIN B ASSAY USING RAT CURATIVE METHOD WITH MODIFIED DIETS AND ORAL ADMINISTRATION OF ADDENDA

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(Received for publication June 24, 1936)

About a year and a half ago it was found necessary in this laboratory to test vitamin B (B_1) fractions more quickly than the rat growth method permitted. The rat curative method as developed by Smith ('30) was considered. Through a personal communication Ammerman and Waterman (data later published in Journal of Nutrition, '35) suggested their modification of the Smith diet, the preparation being simple and practical. It consisted of:

	%
Casein (technical)	16
Osborn and Mendel salt mixture	4
Cod liver oil	2
Unfiltered butter	9
Cornstarch	60
Autoclaved dried bakers' yeast	9

Ammerman and Waterman had started rats weighing 100 to 125 gm. on this diet but in order to shorten the method in our laboratory rats 70 to 75 gm. in weight were used. Over 50% of the animals died without developing polyneuritis. In looking over the literature it was found that Sandels ('30) had used a basal diet containing 15% of bakers' yeast (autoclaved 3 hours at 125°C.). The rats on the basal diet alone died within 25 to 40 days rarely showing symptoms of polyneuritis while the rats on this diet plus 0.2 and 0.4 gm. of yeast or the alcoholic extracts from 0.4 to 0.8 gm. of whole wheat had all developed polyneuritis. Sebrell and Elvove

('31) had produced polyneuritis on a diet containing 15% autoclaved brewers' or bakers' yeast (autoclaved 2½ hours at 15 pounds pressure) and Heyroth ('32) developed polyneuritis on a diet containing 10% Harris' autoclaved brewers' yeast, but he had obtained rats from an outside commercial source. Birch and Harris ('34) had used 10% autoclaved brewers' yeast plus 0.4% brewers' yeast in the diet to produce polyneuritis.

The bakers' yeast in the Ammerman and Waterman diet was autoclaved for 6 hours at 15 pounds pressure. As Sandels' rats on 15% bakers' yeast autoclaved at 125°C. for 3 hours died within 25 to 40 days with no polyneuritis, it was assumed that the unextracted casein and unfiltered butter fat contained the only traces of vitamin B in the Ammerman and Waterman diet. At this time there were some rats in the laboratory on the Sherman and Spohn as modified by Chase ('31) diet plus a vitamin B extract supplying about one-half the vitamin B requirement for protection and gain of 3 gm. per week. Approximately all of these rats developed polyneuritis. It was decided to add to the Ammerman and Waterman diet an amount of yeast supplying about one-third of the vitamin B requirement for growth of 3 gm. weekly. To 100 pounds of diet 400 gm. of brewers' yeast[1] (containing 13.3 Sherman vitamin B_1 units per gram) were added. The food intake of rats from 30 to 50 days on this diet averaged 2.9 gm. daily (maximum amount eaten 3.8 gm., minimum 2.0 gm.). As the diet contained 0.12 Sherman vitamin B unit per gram the animals received 0.35 Sherman vitamin B unit daily (maximum 0.46 unit, minimum 0.26 unit) or approximately one-third of the vitamin B requirement for protection and gain of 3 gm. in weight per week. Rats were run on this diet, the Smith diet and the Ammerman and Waterman diet at the same time.

The incidence of polyneuritis was much greater on the modified diet as is shown in table 1. There was only 31.1% discards on the modified diet as against 54.8 on the Smith diet and 55.7 to 58.1 on the Ammerman and Waterman diet. In a

[1] Dried dehydrated yeast from Northwestern Yeast Company, Chicago, Illinois.

few cases, the rats had four, five or six polyneuritic attacks and as many as nine or ten on the modified diet. The next problem was to prove that the assay run on these polyneuritic rats with oral administration of the addenda gave comparable results with those obtained on the Sherman growth method.

The rats were not used for test until they reached the spastic stage of polyneuritis—a circular movement or a rolling over of body upon being rotated by the tail. In many

TABLE 1

Incidence of polyneuritis on different diets

DIET	TOTAL NUMBER OF RATS USED	WEIGHT OF RATS WHEN PLACED ON DIET	PER CENT RATS DEVELOPED POLYNEURITIS	NUMBER OF DAYS BEFORE POLYNEURITIS DEVELOPED	WEIGHT OF RATS ON DEVELOPING POLYNEURITIS	NUMBER OF POLYNEURITIC ATTACKS PER RAT
		gm.				
Smith	113	50 to 60	45.2	40	57	2.2
Ammerman and Waterman	131	90 to 110	44.3	39	72	1.8
Ammerman and Waterman	184	55 to 85 (Av. 66)	41.9	36	56	2.7
Modified diet (0.12 Sherman vitamin B unit per gram)	620	55 to 85 (Av. 64)	68.9	41	63	2.7
Modified diet (yeast withdrawn as soon as one or more rats became polyneuritic)[1]	200	55 to 85	54.0			2.1

[1] We attempted putting only the polyneuritic test animals on the yeast-free diet, but where there are several hundred rats running at the same time this is well nigh an impossible task.

cases due to this severe condition it took 48 to 72 hours for the rats to be actually cured; gradually improving and gaining weight in the meantime. The condition of each rat was recorded. If the dose were not adequate and the rat remained polyneuritic, Ammerman and Waterman put the rat on test again within 24 hours—in our laboratory 48 hours were allowed to elapse between feedings and 72 to 96 hours with borderline levels where there had been a corresponding

gain in weight. Fewer individual variations occurred when the animals were not started too soon, that is, before polyneuritis was sufficiently pronounced. The 4-day curative period has been the criterion for these tests, the actual duration of cure which the English workers used having been considered more variable due to Heyroth's ('32) findings.

The material was fed by a 1 cc. tuberculin syringe with the needle removed. It was inserted into the mouth of the rat—as far back into the throat as it would reach. The amount delivered was checked by weight. If the material was soluble in water a solution was made and a dosage from 0.1 to 0.2 cc.—preferably 0.1 cc.—was given. If the material was insoluble it was made into a soft paste with fuller's earth and vegetable oil and this was fed in the same way. A paste, such as yeast and water, works very nicely with the tuberculin syringe. In the case of a paste the specific gravity must be taken and the dose in cubic centimeters corrected by the specific gravity factor.

Using the International vitamin B adsorbate as a standard in the rat curative method, the potency of the unknown in Sherman and International vitamin B units was worked out by the formulae:

$$\frac{\text{Curative dose of International standard}}{\text{Curative dose of unknown}} \times (100 \text{ International vitamin B units per gram in standard})$$

$$\frac{\text{Curative dose of International standard}}{\text{Curative dose of unknown}} \times (133 \text{ Sherman vitamin B units per gram in standard})$$

The curative dose is that dose which effects a cure in 80% or more of the animals; the curative dose of the unknown is that which produces the same percentage of cures as the standard. The factor 133 in the second formula is the potency of the International adsorbate in Sherman units which we get in this laboratory using the Sherman rat growth method—it is a conservative figure as some laboratories report as high as 200 to 300.

The rat curative method has been used in assaying many types of material such as water-soluble concentrates, insoluble adsorbates, yeasts of greater potency than 40 Sherman units

TABLE 2

Comparison of results in vitamin B assay using rat growth and rat curative methods

A. Rat growth method

NUMBER OF RATS	DOSE	GAIN IN WEIGHT PER 28 DAYS
	Unknown	
	mg.	*gm.*
8	4.0	—17 (polyneuritic)
7	8.0	+15
7	12.0	+29
	International vitamin B adsorbate	
7	5.0	— 5
7	7.5	+14
7	10.0	+25
	Negative controls	
7		Died within 25 to 40 days without developing polyneuritis

Results: Slightly more than 125 Sherman vitamin B units per gram ($\frac{1000}{8.0}$ or $\frac{7.5}{8.0} \times 133$). Slightly more than 83 International vitamin B units per gram ($\frac{10.0}{12.0} \times 100$).

B. Rat curative method

DOSE	CURED	NOT CURED	PER CENT OF CURES
	Unknown		
mg.			
15	2	4 (2 died within 24 hours—discarded)	50
20	5	1	83
25	6	0	100
	International vitamin B adsorbate		
15.7	3	5	38
18.4	5	2 (1 died within 24 hours—discarded)	83
21.0	5	1 (severe, died within 48 hours—discarded)	100

Results: 122 Sherman vitamin B units per gram ($\frac{18.4}{20.0} \times 133$); 92 International vitamin B units per gram ($\frac{18.4}{20.0} \times 100$).

per gram, and vitamin paste mixtures. Where a finished report in Sherman units was required a check assay using the rat growth method was run as is shown in table 2.

Two levels close together—15.7 mg. and 18.4 mg.—were run on the standard to make interpretation of results more accurately. If the rats were in a severe condition the percentage of cures on the 15.7 mg. level was below 80; if the

TABLE 3

Vitamin B assay using rat curative method with minimum number of animals on unknown

Rat curative method

DOSE	CURED	NOT CURED	PER CENT OF CURES
		Unknown	
mg.			
1.9	0	5	0
2.2	0	3	0
2.5	0	5	0
2.8	0	3	0
3.5	1	4	20
4.0	2	1	67
5.0	2	2 (severe condition)	50
6.0	5	0	100
7.0	4	0	100
		International vitamin B adsorbate	
12.2	2	4	33
15.7	3	4 (1 died within 24 hours—discarded)	50
18.4	9	4 (2 died within 24 hours—discarded)	82

The results on the 5.0 mg. level were discarded and the potency reported between 408 and 612 Sherman vitamin B units per gram ($\frac{18.4}{6.0} \times 133$ and $\frac{18.4}{4.0} \times 133$) or between 307 and 460 International vitamin B units per gram ($\frac{18.4}{6.0} \times 100$ and $\frac{18.4}{4.0} \times 100$).

rats had been started too soon the percentage was more than 80. If a minimum number of rats were used—three or four rats to a level—variable results were obtained as is shown in the assay on a research sample in table 3.

Over 325 samples have been tested in this laboratory by the modified Smith rat curative method with the oral admin-

istration of the addenda. On approximately 10% of these samples which required a report in Sherman units a check assay using the rat growth method was run. The percentage of difference between the two methods was from 2.1 to 24.4 —average 9.1.

DISCUSSION

The number of discards in this laboratory on the Smith diet and the Ammerman and Waterman diet seems higher than reported by others. Possibly it is entirely dependent on the animals used. Ammerman and Waterman claim their animals on the breeders' diet reach the weight of 100 to 125 gm. between 40 and 55 days—in this laboratory it takes 55 or more days for our rats to reach this weight. The disadvantage of using heavier rats is the time item. If higher results are obtained on a diet containing a definite amount of vitamin B this can be obviated by running a standard at the same time which is as necessary in the Sherman rat growth method as in the Smith rat curative method. In comparing one set of results obtained on the rat growth method in this laboratory with results in another laboratory we found that our growth rate was much lower than the other laboratory, but the final results worked out by the formula using the International adsorbate as a standard were the same. In this laboratory we have gotten consistently for the past 3 years only 133.3 Sherman vitamin B units per gram on the International standard adsorbate, thus making our conversion factor from International to Sherman units 1.33 where others got it at 2 or 3; Waterman and Ammerman ('35) gave approximately 2. This shows how impossible it is for all laboratories to use one and the same conversion factor in any bio-assay.

CONCLUSION

The Smith rat curative method with a modified diet is practical and within the limits of accuracy for bio-assays if the following points are adhered to: 1) the standard must be run at the same time as the unknown; 2) a certain critical

judgment is necessary in taking rats definitely polyneuritic; 3) the number of animals should not be less than that used in the rat growth method, six or more rats to each level and not less than two levels; 4) where an animal is used repeatedly for test, 48 hours should elapse between feedings even if the animal becomes polyneuritic within 24 hours; on borderline levels with a corresponding gain in weight 72 to 96 hours are necessary; and 5) it is better to make the interpretation of curative dose at a level where 80% or more of the rats are cured; at borderline levels of less than 80% cures there will be greater individual variations. Until the individual laboratory has developed its technic sufficiently it is advisable to run check assays with another familiar method such as the rat growth method or pigeon maintenance method.

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THE EFFECTS OF GLUCOSE, FRUCTOSE AND GALACTOSE ON KETOSIS, PRODUCED BY ANTERIOR PITUITARY EXTRACT AND BY PANCREATECTOMY

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TWO FIGURES

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Evidence is growing that the different hexoses do not have equal physiological values. C. Voit as early as 1891 found that glucose and fructose were better glycogen formers in the dog than galactose, and this was confirmed by Weinland in 1899. Lusk ('15) reported that the dynamic effect of glucose, sucrose and fructose increased in the order named when given in equal doses to normal dogs. Galactose and lactose lagged behind, the former possibly because of its slower conversion after absorption, the latter because it was not readily digested. More recent evidence places galactose at the head of the list of hexoses in the order of their convertibility to glucose in the phlorhizinized dog (Deuel and Chambers, '25) and fructose ahead of glucose in combustibility (Hornemann, '23). Galactose at certain stages of absorption also leads in rate of glycogen formation (Deuel, MacKay et al., '33) in the normal dog.

As regards the ketolytic action of different sugars Goldblatt ('25) concluded from experiments on fasting human subjects that glucose, fructose, sucrose and maltose were effective while galactose, mannose, lactose (and glycerol) were not.

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Producing ketosis in man in the same manner and also by means of a protein-fat diet, Deuel, Gulick and Butts ('32) found galactose possessed a greater lytic action than glucose. Ketonuria was depressed farther and maintained at a lower level for a longer period of time. Fructose also surpassed glucose in its ketolytic effect after fasting and appeared to be intermediate between the other two hexoses.

Butts ('34) by experiments on rats in which ketosis was produced by fasting and by administration of sodium acetoacetate found galactose most effective, lactose next and glucose least, in controlling ketonuria. Water intake was especially high after galactose.

Shapiro ('35) fed *l*(+)-lactic acid, pyruvic acid and glycerol, as well as glucose, in amounts demonstrated to be glycogenic, and found a definite ketolytic action in rats fed diacetic acid. On the other hand *d*(—)-lactic acid, acetaldehyde, ethyl alcohol and ethylene glycol did not show any appreciable glycogen formation and very little ketolytic action. She found that a definite relationship existed between the ability of a substance to form glycogen and its ketolytic action.

Chaikoff and Weber ('27) concluded that carbohydrate had no antiketogenic action in depancreatized dogs in complete absence of insulin. Selle ('27) held that administration of glucose to patients in diabetic coma often relieved the symptoms of ketonuria; but glucose had no effect on ketonemia of animals deprived of their pancreas.

The present study was suggested by the thought that pancreatic ketonuria alone may not be an adequate criterion of ketogenesis and that the comparative effects of the different hexoses should be studied in ketosis produced by still other means. Dogs were chosen as subjects and ketosis produced 1) by high fat diets supplemented by injection of the ketogenic hormone of the anterior pituitary, and 2) by pancreatectomy.

EXPERIMENTAL

Normal female dogs were fed 1 pint of cream daily for several weeks prior to the studies with the sugars. When a

measurable ketosis had been established administration of anterior pituitary extract was begun.

Female dogs were completely depancreatized under ether and morphine anesthesia. Subcutaneous injections of insulin were given twice daily until the wound was entirely healed. Insulin was then withdrawn until a satisfactory D : N ratio was established, which, by experiment, was found to reflect about maximum ketosis. This usually occurred on the fourth to fifth day.

The dogs were confined in metabolism cages throughout the entire period of the experiment. A mixture of toluene and thymol was used to preserve the urine. Water was given ad libitum.

Respiratory quotients were determined, blood samples were taken from the saphenous vein and catheterizations were made, in the order mentioned, at approximately 8.00 A.M., 12.00 NOON, 4.00 P.M. and 8.00 P.M. The exact times were always noted and all values calculated to the basis of 1 hour.

The first 4-hour period (8.00 A.M. to 12.00 NOON) was used as a control. Fifty grams of the sugar to be tested were given after the 12.00 NOON catheterization and the effects on the ketosis and metabolism were studied in the afternoon. The sugar was dissolved in 100 cc. of water and administered by means of a stomach tube. To the normal dog on a high fat diet, 10 cc. of a crude pituitary extract, prepared by the method of Magistris ('32) and containing the diabetogenic and fat metabolism hormones, were injected subcutaneously at the same time as the sugars were given. The diet of the depancreatized dog consisted of ground beef steak, 20 gm. pancreatin, 40 gm. of lard and 10 gm. of bone ash. Feeding always occurred at 8.00 P.M.

Autopsies were performed on all the depancreatized dogs at the completion of the experiments to determine whether any residual pancreatic tissue remained.

Pfanstiehl C.P. galactose and fructose and C.P. glucose furnished by the Corn Industries Research Foundation were used.

The gaseous metabolism determinations were carried out with the open circuit type of respiration apparatus (Tissot). The dogs were trained to lie still with a face mask and breathe into the spirometer. The analyses of oxygen and carbon dioxide in the expired air were made on a Haldane gas analyzer.

After the withdrawal of the blood a filtrate was made as soon as possible by the method of Folin and Wu. The blood sugars were determined by Benedict's method ('28). Urinary nitrogen was determined by the usual macro Kjeldahl method, using alizarine as the indicator. Urinary sugars were determined by a combination of the Munson and Walker and Bertrand methods (Mathews, '25). Blood acetone bodies were determined by the Shaffer-Hubbard distillation method as adapted by Behre and Benedict ('26) employing, however, Hubbard's method of titration. Acetone plus diacetic acid were determined together. Urine acetone plus diacetic and β-hydroxybutyric acid and blood β-hydroxybutyric acid were determined by the method of Hubbard ('21). The yield of acetone from β-hydroxybutyric acid by the above method was approximately 75%. No correction was made for the 25% which was not determined. This error appears to be practically constant.

RESULTS

High fat diet and anterior pituitary extract

As many investigators have shown, it is very difficult to produce a marked ketosis in a normal adult dog maintained on a high fat diet. This was accomplished, however, by use of injections of anterior pituitary extract (Magistris, '32) subcutaneously. This extract contains the diabetogenic and fat metabolism hormones which Anselmino and Hoffman ('34) recently showed to be two separate entities. The diabetogenic hormone probably lowers carbohydrate oxidation by suppressing the secretion of insulin (Képinov et al., '34). Anselmino and Hoffman ('31) and others, have shown that the fat metabolism hormone increases fat metabolism and the production of ketone bodies. Thus, by use of these two hor-

mones, a condition was obtained which closely simulates diabetes. The condition resulting from the injection of these two hormones was rather transient, but of sufficient duration to satisfy the intended purpose. This is shown in table 1 by a typical example of an experiment, and in figure 1 by averages of a series of experiments.

In the control, receiving extract alone (fig. 1) the average blood sugar rose gradually from 65 to 110 mg. per cent and reached a maximum in 8 hours. By the next morning it had fallen to the basal level. The rise can indicate either that glucose oxidation is suppressed or that the diabetogenic hor-

TABLE 1

High fat diet and anterior pituitary extract. Control

TIME	URINE N	D : N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.			*mg./%*	*mg./%*	*mg./hr.*	*mg./%*	*mg./hr.*
8.00 A.M. basal det.	0.10		0.71	63	30	2	79	9
12.00 NOON basal det.	0.10		0.71	63	29	2	78	8
12.10 P.M.	10 cc. pituitary extract							
4.00 P.M. 1st experimental period	0.19		0.70	73	31	6	88	21
8.00 P.M. 2nd experimental period	0.11		0.68	115	72	3	125	19
8.10 P.M.	Fed 1 pint xx cream							
8.00 A.M. basal	0.10		0.71	68	32	5	78	22

mone stimulates the adrenals, as Heydeman, Duensing and Lucke ('33) believed. It is possible that the maximum change of blood sugar, blood acetone, blood β-hydroxybutyric acid and R.Q., in all of the experiments, was not observed, because of the necessarily long interval between determinations. Concurrently with the rise in blood sugar, the R.Q. dropped slightly from 0.71 to 0.68. The three experiments were closely concordant.

The control curves for blood ketone bodies (fig. 1) closely paralleled the one for blood sugar, making an average increase from 75 to 125 mg. per cent in the case of the β-hydroxybutyric acid and 26 mg. to 73 mg. per cent in the case of the blood

acetone. The urine ketone bodies increased in the controls from 7 to 23 mg. per hour for β-hydroxybutyric acid and from 2 to 7 mg. per hour for acetone; but did not parallel the blood ketone rise. The maximum rise came during the first 4-hour period.

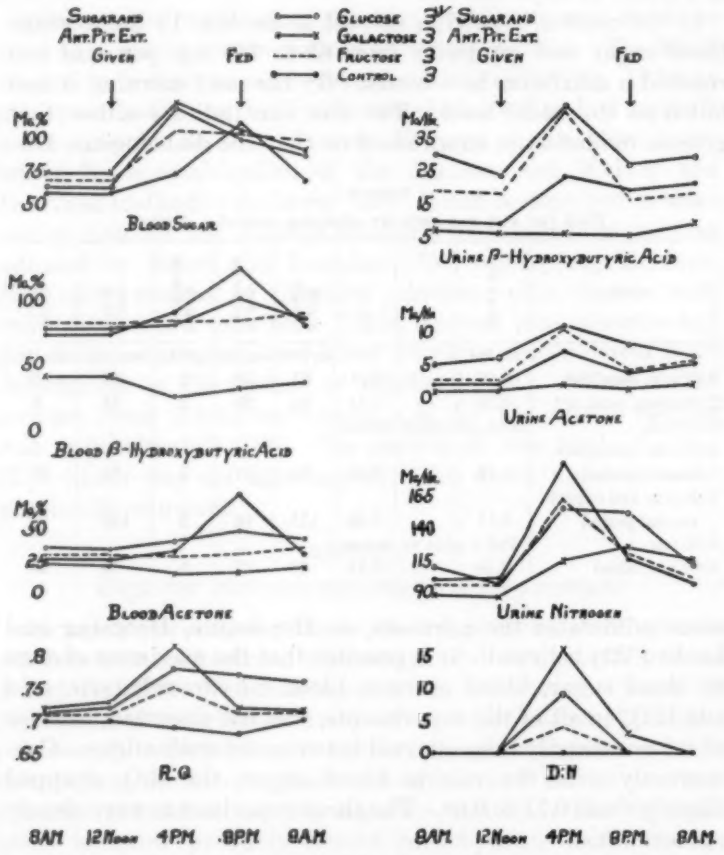

Fig. 1 Changes caused by the simultaneous oral administration of 50 gm. of sugar and subcutaneous injection of anterior pituitary extract to dogs, which were maintained on a high fat diet.

[1] Number of experiments included.

Since the blood ketone bodies remained at approximately the basal level during the first 4 hours, it seemed that the kidney prevented a further increase in the blood ketone bodies by excreting them as fast as they were formed. During the next period the ketone bodies were formed at a faster rate than the kidney was able to excrete them. This resulted in a marked increase in the blood, thus confirming the idea that formation and excretion may differ considerably in rate.

During the first 4-hour period after the injection of the extract, the nitrogen elimination rose from 100 mg per hour

TABLE 2

High fat diet and anterior pituitary extract. Glucose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.10		0.71	64	40	3	79	27
12.00 NOON basal det.		0.09		0.73	64	36	4	75	17
12.10 P.M.	10 cc. pituitary extract + 50 gm. glucose								
4.00 P.M. 1st experimental period	0.90	0.14	7:1	0.79	125	46	11	77	46
8.00 P.M. 2nd experimental period		0.13		0.80	116	51	4	105	21
8.10 P.M.	Fed 1 pint xx cream								
8.00 A.M. basal det.		0.10		0.77	100	40	5	70	27

to 193 mg. per hour (average) and then returned to approximately normal during the subsequent 4-hour period. Only a small part of this rise could be explained by the fact that the extract injected contained 3 mg. of nitrogen per cubic centimeter. Since the diabetogenic hormone suppresses carbohydrate oxidation, the nitrogen sparing action of carbohydrate is lowered. It is also possible that the extract directly stimulates protein catabolism. No glycosuria was observed.

Tables 2, 3 and 4, which are typical examples of individual experiments, and figure 1, which shows averages of a series of experiments, illustrates the effects that glucose, fructose and galactose have on ketosis and the general metabolism of the

dogs. Again the three experiments in each group were closely concordant.

Following the administration of glucose and extract, the blood sugar rose from 65 to 125 mg. per cent during the first

TABLE 3

High fat diet and anterior pituitary extract. Fructose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.09		0.71	69	28	3	85	18
12.00 NOON basal det.		0.09		0.71	70	28	3	85	18
12.10 P.M.	10 cc. pituitary extract + 50 gm. fructose								
4.00 P.M. 1st experimental period	0.44	0.18	2.5:1	0.74	101	28	10	88	49
8.00 P.M. 2nd experimental period		0.11		0.71	103	32	4	88	12
8.10 P.M.	Fed 1 pint xx cream								
8.00 A.M. basal det.		0.09		0.71	103	35	8	96	19

TABLE 4

High fat diet and anterior pituitary extract. Galactose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.09		0.72	68	20	1	36	8
12.00 NOON basal det.		0.09		0.73	68	19	1	35	7
12.10 P.M.	10 cc. pituitary extract + 50 gm. galactose								
4.00 P.M. 1st experimental period	2.79	0.12	17:1	0.83	122	15	2	15	7
8.00 P.M. 2nd experimental period	0.38	0.12	2:1	0.74	91	15	1.5	27	5
8.00 P.M.	Fed 1 pint xx cream								
8.00 A.M.		0.11		0.74	82	18	3	30	11

4-hour period, and then dropped to 100 mg. per cent during the next 4-hour period. The blood sugar curve following the administration of galactose and extract closely paralleled that for glucose. Fructose raised the blood sugar from 65 to only

105 mg. per cent during the first 4-hour period; but, maintained it at this level for the subsequent 4-hour period. All three of the monosaccharides sustained the blood sugar above normal for 20 hours after the administration. Many investigators have found that if a certain class of foods is withheld from the body for a long period of time, the body compensates for the deficiency by adaptation to increased utilization of another class of foods. When the deficiency food is again added to the diet, it requires a certain period of time before the body can readjust itself to utilize this food, which is now a relatively foreign substance to the body (Joslin, '28). Furthermore, in the present instance the oxidative mechanism of carbohydrate has been interfered with by the injection of the diabetogenic hormone. These facts probably accounted for the prolonged high blood sugars.

The injection of 10 cc. of pituitary extract alone raised the blood β-hydroxybutyric acid in 8 hours from 75 to 125 mg. per cent and the blood acetone from 26 to 73 mg. per cent. When glucose was administered along with the extract (table 2 and fig. 1), the blood β-hydroxybutyric acid rose, on the average, in the same time from 77 to 102 mg. per cent and the acetone from 34 to 50 mg. per cent. Fructose (table 3 and fig. 1) completely offset the effect due to the extract. The blood ketone bodies were maintained at approximately a constant level during the entire experiment. Galactose (table 4 and fig. 1), however, not only completely offset the effect due to the extract, but also lowered the blood ketone bodies below the basal level. The blood β-hydroxybutyric acid dropped from 40 to 25 mg. per cent and the blood acetone from 22 to 15 mg. per cent. Both rose to approximately basal level by the twentieth hour. Quite different were the effects of glucose and fructose on the ketonurias. During the first 4 hours after the administration of glucose, the urine β-hydroxybutyric acid rose from 23 to 45 mg. per hour and then returned to the basal level during the next 4-hour period. The high points reached were above the controls. The urine acetone rose from 7 to 11 mg. per hour during the first 4-hour period, and then re-

turned gradually to normal by the twentieth hour. The curves for urine ketone bodies (fig. 1) following the administration of fructose, paralleled closely those for glucose. Galactose held the excretion of β-hydroxybutyric acid to the base level and even caused it to drop slightly by the eighth hour, while the urine acetone increased only slightly during the first 4-hour period. Both returned to basal during the next 4 hours.

Galactose also exerted a greater protein sparing action than did glucose or fructose. This is shown in table 4 and figure 1. The urine nitrogen after galactose administration rose on the average from 85 to 115 mg. per hour during the first 4-hour period. It rose to 127 mg. per hour during the next period and then dropped slightly. Contrast this with the control curve. Fructose exerted a slightly greater nitrogen sparing action than glucose. The nitrogen elimination after the administration of glucose and extract rose from 95 to 155 mg. per hour during the first 4 hours and remained at this level for the next 4-hour period. It returned then to approximately normal during the subsequent 12 hours. After the administration of fructose and extract, the nitrogen elimination rose on the average to 160 mg. per hour during the first 4-hour period and then dropped to 120 mg. per hour during the succeeding 4-hour period.

A slight glycosuria was observed in all the experiments during the first 4-hour period after the administration of sugar. Galactose caused the highest glycosuria followed by glucose and then fructose.

Glucose raised the R.Q. from 0.72 to 0.78 during the first 4-hour period and maintained it above 0.76 for the subsequent 16 hours of the experiment. In the case of fructose, the R.Q. rose from 0.71 to 0.75 during the first period and then returned to basal during the next period. Galactose gave a similar reaction, but the rise from 0.73 to 0.81 was slightly higher at the end of the first 4-hour period. No simple proportionality existed between the effects on combustion and the ketolytic action, as judged by events at these 4-hour intervals.

Depancreatized dog

Tables 5, 6 and 7, and figure 2, show the effects of administration of glucose, fructose and galactose on depancreatized animals. The tables illustrate typical examples of individual experiments, while the figure shows the average percentage

TABLE 5

Depancreatized dog. Glucose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	*gm./hr.*			*mg./%*	*mg./%*	*mg./hr.*	*mg./%*	*mg./hr.*
8.00 A.M. basal det.	4.62	0.87	5:1	0.76	285	26	6	50	113
12.00 NOON basal det.	0.38	0.43	0.9:1	0.72	228	24	9.5	42	31
12.10 P.M.	50 gm. glucose								
4.00 P.M. 1st experimental period	3.79	0.24	18:1	0.72	392	17	9	42	23
8.00 P.M. 2nd experimental period	6.50	0.24	27:1	0.71	285	21	16	34	38
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	4.64	0.88	5:1	0.76	205	26	26	46	92

TABLE 6

Depancreatized dog. Fructose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	*gm./hr.*			*mg./%*	*mg./%*	*mg./hr.*	*mg./%*	*mg./hr.*
8.00 A.M. basal det.	4.2	0.84	5:1	0.77	286	25	28	50	116
12.00 NOON basal det.	0.89	0.53	2:1	0.73	286	25	11	54	41
12.10 P.M.	50 gm. fructose								
4.00 P.M. 1st experimental period	2.36	0.29	8:1	0.72	412	24	12	55	38
8.00 P.M. 2nd experimental period	5.01	0.25	20:1	0.72	354	28	9	58	26
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	3.56	0.63	5.5:1	0.75	281	26	22	55	96

change in a series of experiments which were fairly concordant. Computation of percentage change was used because there existed a considerable difference in the severity of the dogs' conditions following the withdrawal of insulin.

The dogs used were all good eaters, very active, friendly and in the best of health. A few of the dogs which refused food after the operation were discarded because they declined rapidly in general nutritive condition.

The curves for blood sugars (fig. 2) were practically identical for the three hexoses. They rose to practically the same level at 4 hours and then dropped to approximately normal by the eighth hour. Actually the maxima must have come earlier and may not have coincided so closely.

The excretion of sugar in the urine after the administration of glucose and fructose was highest during the second 4-hour

TABLE 7

Depancreatized dog. Galactose

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	*gm./hr.*			*mg./%*	*mg./%*	*mg./hr.*	*mg./%*	*mg./hr.*
8.00 A.M. basal det.	4.02	0.88	5:1	0.76	294	34	20	76	103
12.00 NOON basal det.	0.58	0.45	1.3:1	0.72	303	40	11	76	54
12.10 P.M.	50 gm. galactose								
4.00 P.M. 1st experimental period	7.44	0.48	16:1	0.72	440	38	15	70	50
8.00 P.M. 2nd experimental period	4.97	0.40	13:1	0.71	354	29	15	54	48
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	4.28	0.85	5:1	0.76	286	25	28	50	116

period (tables 5 and 6) while the maximum excretion, following galactose administration, was observed during the first 4-hour period (table 7) thus indicating that galactose is absorbed faster (Cori, '25 a) and has a lower renal threshold (Shay et al., '31; Rowe and Chandler, '24; Folin and Berglund, '22). Only 80% of the administered sugar was recovered during the 20-hour period. The remaining 20% probably diffused into the tissues (Cori and Goltz, '25) and was gradually returned to the blood stream and excreted. If the urine had been collected for another 24 hours probably nearly all of the administered sugar would have been regained.

The D:N ratios fluctuated considerably from period to period. This variation is very characteristic of a depancreatized dog. They were usually about 5:1 in the early morning which was much higher than the Minkowski ratio of 2.8:1. This was due to the fact that 20 gm. of pancreatin, containing about 80% dextrin was given along with the ground

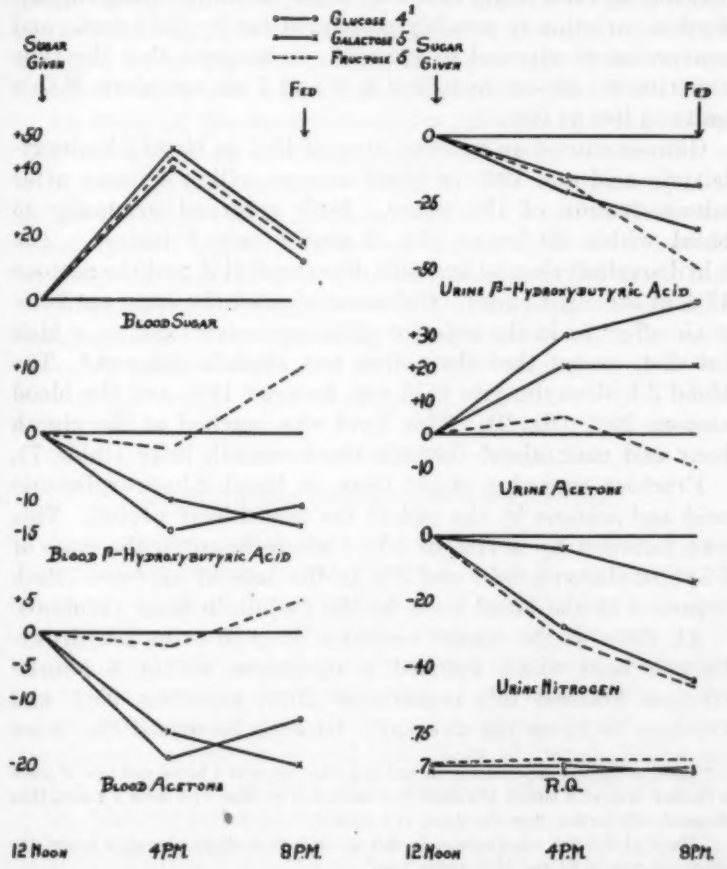

Fig. 2 Percentage change caused by the oral administration of 50 gm. of sugar to depancreatized dogs. All curves represent averages of the experiments.

[1] Number of experiments included.

beef steak and lard the preceding evening. When a correction was made for this extra sugar, the resulting D:N ratio was about 3.5:1, which is within the range of daily variation of a depancreatized dog.

None of the sugars was oxidized as is shown by the R.Q. which usually dropped gradually from 0.71 or 0.72 to 0.68 or 0.69 during the 8 hours following sugar administration (fig. 2). Such a variation is possibly accounted for by the ketosis and conversion of glycerol to glucose. It happens that the type experiments shown in tables 5, 6 and 7 do not show R.Q.'s quite so low as this.

Glucose caused an average drop of 15% in blood β-hydroxybutyric acid and 19% in blood acetone within 4 hours after administration of the sugar. Both returned gradually to basal within 20 hours (fig. 2 shows only 8 hours). The β-hydroxybutyric acid was still depressed 11% and the acetone 13% at the eighth hour. Galactose showed the same antiketogenic effect as in the anterior pituitary extract dog on a high fat diet, except that the action was slightly delayed.[2] The blood β-hydroxybutyric acid was lowered 14% and the blood acetone 20% (fig. 2). This level was reached at the eighth hour and maintained through the twentieth hour (table 7).

Fructose caused a slight drop in blood β-hydroxybutyric acid and acetone by the end of the first 4-hour period. This was followed by a rise of 10%[3] above basal in the case of β-hydroxybutyric acid and 5% in the case of acetone. Both returned to the basal level by the twentieth hour (table 6).

All three of the sugars caused a drop in urine β-hydroxybutyric acid which reached a maximum within 8 hours.[4] Glucose lowered this constituent 20%, galactose 35% and fructose 50%, on the average. Glucose increased the urine

[2] Three of the five experiments showed a marked drop at 4 hours and two of these a further drop at 8 hours; the other two continued at base level until 4 hours, then dropped still farther than the three, at 8 hours.

[3] Three of the five experiments showed no or a very slight rise at 8 hours, the other two rose to 24 and 13% above basal.

[4] Only three individual exceptions to this course—two with glucose and one with fructose all of which showed a return to or toward basal at 8 hours.

acetone plus diacetic 30% during the first 4-hour period and it rose to 40% during the subsequent 4-hour period. Galactose caused a 20% rise in urine acetone plus diacetic during the first 4-hour period and maintained it at this level for the next period. Fructose raised this fraction slightly during the first 4-hour period, but lowered it to 10% below the basal level during the next period. The high urine β-hydroxybutyric acid and acetone observed in the morning was due to the lard which was fed the previous evening. This was not observed when lard was withheld from the diet.

All three of the sugars showed a nitrogen sparing action. Glucose caused an average drop in nitrogen elimination of 44%, fructose 46% and galactose 12%. The maximum sparing action was observed, in all three cases, at the end of the second 4-hour period (fig. 2). Galactose did not show any superior sparing action in the depancreatized dogs as it did in the high fat dogs. Fructose and glucose showed a comparatively equal sparing action. The high urine nitrogen observed in the early morning specimen was due to the food which was given the preceding evening (tables 5, 6 and 7).

DISCUSSION

High fat and anterior pituitary extract

Since glucose, which may be considered the most physiological of the sugars, forms the chief, if not the only reducing sugar of the blood, one would think it ought to have the best antiketogenic power. However, such is not the case. The antiketogenic effect of glucose was quite moderate. Galactose exerted the greatest ketolytic action of any of the three monosaccharides and fructose intervened between the two. These results are entirely in accord with those of Deuel, Gulick and Butts ('32), who worked with humans and those of Butts ('34) who worked with rats.

Most investigators have used the change in ketonuria as their criterion for determining the ketolytic action of various substances. The writers do not think this is an adequate way

to determine such an effect because it was found that the amount of ketone bodies in the urine varied often in the same direction as in the blood, but sometimes also in the opposite direction. This was well shown in the determinations exhibited in tables 2, 3, 4 and fig. 1. No positive explanation for the peculiar rise in urine ketone bodies after the administration of glucose and fructose can be offered. It is not likely that it is wholly a washing out phenomenon due to the diuresis which is known to accompany glycosuria; otherwise it would certainly have been observed in the depancreatized dog (fig. 2) after the ingestion of sugar. Hubbard and Wright ('24) have shown that the variation in excretion of ketone bodies does not depend upon variations in urine volume. A considerable rise was observed in the control experiments; therefore, the effect may be due entirely to the extract.

Why galactose has the best antiketogenic effect is a matter of conjecture rather than demonstration in these experiments. Deuel et al ('32) claim that it is due to the fact that galactose maintains the tissue sugar at a higher level for a longer period of time than either glucose or fructose, and also that it is a better glycogen former than was previously supposed. We have no evidence that this was the case in the experiments under discussion because liver and tissue glycogen and the concentration of sugar in the tissues before and after administration, were not determined.

The sugars seem to act in proportion to their diffusibility. It is well known that the renal threshold is much lower for galactose than the other sugars, and that it is most readily absorbed from the intestine. Glucose is next in the order of absorption and fructose last (fig. 1, D:N). In view of this, galactose probably arrives at the site of ketone body production (Macallum, '30) most rapidly, followed by glucose and fructose in that order. Whether they act in order of combustibility or power of combination to decrease ketosis is not yet certain.

Depancreatized dogs

With regard to antiketogenic effects in the depancreatized dogs, the findings were particularly astonishing in the light of some of the other work in this field. Chaikoff and Weber ('27) found that glucose had no effect on ketone body excretion in depancreatized dogs and Selle ('27) came to the same conclusion using the blood ketone bodies as his criterion. The present experiments show that glucose exhibited a ketolytic action, in spite of the fact that the sugar was not oxidized.

According to Shaffer's theory of antiketogenesis, carbohydrate has to be oxidized before the formation of ketone bodies is interfered with. This cannot be applied to the results under discussion because there was no oxidation of carbohydrate as indicated by the R.Q. Intermediate determinations bear out this statement.

From these experiments, one can rightfully say that carbohydrate oxidation is not the only explanation of an antiketogenesis. In another communication (Murlin, Nasset, Murlin and Manly, '36) from this laboratory, it is shown that the administration of sugar lowered the ketosis markedly in human subjects on high fat diets, in some instances reducing it almost to the vanishing point even when the R.Q. did not rise appreciably. The authors are of the opinion that under these circumstances the sugar went to fill up the depleted glycogen stores and was not burned. This phenomenon is also well exemplified in the experiments where the dogs on the high fat diet were given galactose (fig. 1). At the eighth hour after the administration of galactose, the average R.Q. had returned to the basal level of 0.72, indicating that very little, if any, sugar was being oxidized at this determination. A marked ketolytic action, however, was observed between 8.00 P.M. and 8.00 A.M. On the other hand, at the eighth hour after glucose administration, the R.Q. indicated that sugar was still being oxidized, yet there was a much smaller ketolytic effect.

Probably a close relationship exists between the rate of glycogen formation from the three monosaccharides and the ketolytic action which they exert. The antiketogenic effect

following the ingestion of glucose reached a maximum about the fourth hour and then gradually returned to normal. Deuel et al. ('33) found that the liver glycogen of dogs was greater 6 hours after administration of glucose than it was in animals which retained a corresponding amount of galactose; however, the liver glycogen in animals killed at intervals of 12 to 72 hours after sugar feeding, was higher in the dogs which had been fed galactose. This hexose caused a gradual lowering of ketone bodies which reached a maximum about the eighth hour.

Experiments on glycogen formation in the dog cannot be accomplished satisfactorily in short periods.

Cori ('26) also Feyder and Pierce ('35) have shown that fructose forms liver glycogen at a faster rate than glucose. When determinations were made 2 hours after the ingestion of fructose in the present work, a marked ketolytic effect was observed, which returned to the basal level by the fourth hour; but sufficient evidence has not been accumulated to warrant a definite statement that the maximum always occurs at this time.

Many authors are of the opinion that the depancreatized dog is capable of forming some glycogen, at least temporarily. Shapiro ('35) has shown that only glycogen formers exerted ketolytic action in the rat. It appears, therefore, that the formation of glycogen may be an important factor in the control of antiketogenesis in these experiments. In the present work it was considered more important to compare effects of different sugars on the same dog than to sacrifice them for glycogen and tissue sugar.

Deuel et al. ('32) claimed that galactose maintained the tissue sugar at a higher level, for a longer period of time, than either of the other two monosaccharides. It is not likely that this could apply to these experiments, because glucose, which had very little antiketogenic effect in the high fat dog, exerted a ketolytic effect equal to or slightly greater than galactose in the depancreatized dog. Yet the blood sugar curve for the three monosaccharides closely paralleled each

other and galactose was eliminated more rapidly than the other two.

The fact that galactose and glucose are aldehydes and fructose is a ketone, may be the reason for their greater antiketogenic effects in the depancreatized dog even though all are converted to glucose eventually. Ringer ('14), Shaffer ('21) and others, believe that it is the aldehyde group which combines with the ketone bodies in such a way that they are rendered oxidizable. Since glucose exhibited very little antiketogenic effect in the dog fed on a prolonged high fat diet and given anterior pituitary extract, such an explanation would not hold for this animal. However, it is possible that the depancreatized dog is capable of oxidizing a certain percentage of ketone bodies in its system when a large amount of an aldehyde sugar is present. Joslin ('28) has suggested that the depancreatized dog is capable of producing something which brings about a certain chemical relationship between the ketone bodies and the aldehyde sugars. This enables the depancreatized dog to oxidize the ketone bodies to a much greater extent than does a diabetic man. The increased carbohydrate concentration in the tissues may also have exerted a fat sparing action which is the basis of Macallum's ('30) theory of antiketogenesis.

The urine β-hydroxybutyric acid in this set of experiments reflected very well the blood changes except in the experiments where fructose was administered. At the eighth hour after fructose was given, the urine β-hydroxybutyric was markedly lowered, while the blood β-hydroxybutyric was above the basal level. This seems to indicate that there was a retention of the acid by the kidney.

Since galactose in these experiments did not show as superior a nitrogen sparing action as it did in the high fat dogs, and yet produced a marked lowering of blood ketones, the antiketogenic effect and nitrogen sparing action of the sugars are not necessarily accomplished by the same mechanism.

CONCLUSIONS

1. Galactose has been demonstrated to possess a greater ketolytic action than glucose in the dog, on ketosis due to a prolonged high fat diet and injection of anterior pituitary extract. Fructose was intermediate. Likewise, the nitrogen sparing action falls in the same order, galactose, fructose, glucose.

2. Glucose and galactose possessed approximately equal ketolytic action in the depancreatized dog—glucose being slightly better, although neither was oxidized.

3. Fructose possessed no ketolytic action in the depancreatized dog up to 8 hours following ingestion.

4. The nitrogen sparing action of fructose and glucose were greater than that of galactose in the depancreatized dog.

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COMPARATIVE EFFECTS OF GLUCOSE, SUCROSE AND FRUCTOSE ON KETONE SUBSTANCE PRODUCTION IN PHLORHIZINIZED DOGS

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TWO FIGURES

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INTRODUCTION

Relatively little information is to be found in the literature concerning the comparative effects of different sugars on ketonuria and ketonemia due to phlorhizin. Apparently the only work concerning this problem directly is that of Hornemann ('23) and of Wierzuchowski ('25) who found no difference between glucose and fructose in removing the ketosis of completely phlorhizinized dogs. Wierzuchowski noted also that there was nitrogen sparing action and complete abolition of ketonemia without a compensatory after excretion of the ketone bodies.

Considerably more data are available in reference to the effects of various sugars on ketonuria and ketonemia produced by technics other than phlorhizin. These have been reviewed in the paper preceding this one.

From work on dogs in which ketosis was induced by high fat diet and injection of anterior pituitary extract, Clark and Murlin ('36) demonstrated that a ketolytic order of galactose, fructose and glucose held in this preparation. At the same time they demonstrated that the ketolytic order of the same sugars in totally depancreatized dogs became glucose, galactose and fructose.

[1] The authors are indebted to the Corn Industries Research Foundation for the fellowship, held by them successively, which made this work possible.

The protein-sparing action of sugar in phlorhizinized dogs was noted by Ringer ('12), Benedict ('23) and Gaebler and Murlin ('25). A protein sparing action for galactose, fructose and lactose was noted by Deuel and Chambers ('25), and Wierzuchowski ('27) demonstrated that it ran parallel with decrease in ketonuria. The need for simultaneous consideration of nitrogen sparing action in conjunction with ketolytic action was demonstrated by Shapiro ('35) in her work on sodium aceto-acetate fed rats. She found that ethyl alcohol gave no ketolytic effect when the nitrogen excretion was within the normal limits, but in certain instances when the urinary nitrogen was high, there was a decrease in ketonuria. This was assumed to be due to a stimulation of the nitrogen metabolism by the alcohol and the ketolytic action of the extra protein burned, an effect noted by Benedict and Osterburg ('15) and others.

It was the purpose of the present work to investigate the relative effects of the sugars, glucose, fructose and sucrose on the ketosis of completely phlorhizinized dogs.

EXPERIMENTAL

Female dogs in which phlorhizin diabetes was produced by the daily subcutaneous injection of recrystallized phlorizin suspended in sterile olive oil were used as subjects. The results to be reported are based on two series of experiments, the first in which phlorizin diabetes was induced according to the method of Sansum and Woodyatt ('15) using epinephrine at 3-hour intervals beginning with the inception of phlorizinization, and the second in which the injection of epinephrine was modified to two injections on the third day of phlorizinization. In both series phlorhizin injections were given prior to feeding at approximately 8.00 P.M. and the procedure started 4 days before the beginning of an experiment. In the first series the dogs were fed a calculated amount of food proportional to the surface area formula of Cowgill and Drabkin ('22) in an attempt to equalize the degree of ketosis in dogs of different weights. This was abandoned in

the second series, all dogs being fed 250 gm. of ground meat, 5 gm. of bone ash and 15 gm. of lard.

During the progress of an experiment, the animals were kept in metabolism cages from which the urine could be collected quantitatively. Water was given ad libitum.

As will be pointed out later, the method of conducting an experiment differed in the two series. Individual experimental days, divided into 4-hour periods beginning at 8.00 A.M. and ending at 8.00 P.M., were the same in both series. At the end of each 4-hour interval urine samples were obtained by catheterization, the exact time being noted so that rate calculations could be made, and blood samples were obtained from the saphenous vein. On each urine sample determinations of nitrogen (Kjeldahl) and sugar (Munson and Walker) were made. In the first series beta-hydroxybutyric acid and acetone were determined by Hubbard's ('21) method. In the second series Van Slyke's ('17) method for total acetone was substituted for the Hubbard method. Blood sugar was determined by Benedict's ('28) method. Beta-hydroxybutyric acid and acetone in the blood were determined in the first series by the Shaffer-Hubbard distillations as adapted by Behre and Benedict ('26), but using the Hubbard titration instead of the colorimetric method. In the second series Van Slyke and Fitz's ('19) method was used in determining total acetone.

The sugars used in this experiment were d-glucose supplied by the Corn Industries Research Foundation, sucrose and fructose C.P. Pfanstiehl. In the first series glucose and sucrose were given in doses of 50 gm. in 400 cc. of water by stomach tube immediately following the 12.00 M. sample. In the second series fructose and glucose were given in the same way but successively in doses of 25 and 15 gm. in 150 and 100 cc. of water, respectively.

RESULTS

Before presenting the effects of the different sugars it is necessary to explain the terms used in the tables. Changes in ketonuria can hardly give the whole picture of any ketolytic effect, for the reason that while a certain amount of ketone

bodies is being excreted the level in the blood may be changing. Nor would the change in ketonemia alone give expression to the entire ketolytic effect. Ketone level in the blood expressed in terms of milligrams per cent denotes merely a certain fullness, as one might speak of railroad cars as full, half full, quarter full, etc. To get the total amount in transit one must know the number of cars, or, as in the present instance, the blood volume.

Having obtained the blood volume[1] by the vital red injection method (Peters and Van Slyke, '32) total ketosis, representing the amount of ketone bodies in circulation at any given time, is obtained as the product of the blood volume and the per cent concentration of ketone bodies as determined. Standing alone these figures are not significant of the true ketone body production of the animal, since no account is taken of the amount that is excreted in the urine. Since at body temperature ketone bodies are completely soluble, it is assumed that there will be an equilibrium between the blood and the tissues with respect to these substances, and that the kidneys fairly promptly will excrete all that reaches the organs since there is apparently no threshold value. Total ketosis and ketonuria are combined by taking the change in total ketosis from period to period, representing either an increase or a decrease, reducing this change to terms of acetone formed or burned per hour by dividing by the time interval, and adding this to the milligrams of acetone excreted in the urine per hour. Calculated according to the formula

$$\frac{\Delta \text{ total ketosis in mg.}}{\text{time in hours}} + \text{urine ketones in mg./hr.}$$

the result is called ketone substance production and represents more nearly the amount of ketone substances formed in the animal in any given interval of time.[2] The ketolytic effect

[1] In more than two-thirds of the animals. In the others blood volume was taken as 11% of the body weight.

[2] It is realized of course that this method of calculation overlooks the ketone substances which may not yet have reached the blood stream. While inclusion of the muscular mass and liver at, say, 50% of the body weight, would give larger numbers, the uncertainty of equal distribution in all this mass as compared with blood volume would be greatly increased.

of the different sugars, then, is based on ketone substance production as will be demonstrated in the following tables.

Variation in ketone substance production

It has been established from the control experiments in each series that there is considerable variation in the ketosis developed. This variation seems to be dependent upon a number of conditions, any one or all of which may play a part in a given experiment. Among those conditions are included the number of times an animal has been used for experimental work. Dogs phlorhizinized but once develop a higher degree of ketosis than dogs used several times. Apparently a degree of tolerance is developed by which the animal is able to eliminate or burn ketone bodies which reach a certain level in the blood. Goldfarb and Himwich ('33) have suggested that ketone substances may be oxidized in the presence of lactic acid. This implies that the degree of tolerance is dependent upon the amount of lactic acid present in the tissues. Another factor seems to be the nutritional state of the animal. It has been shown by Junkersdorff ('25) that ketonuria is more intense in well-fed phlorhizinized dogs than in fasting dogs. As has already been stated, an attempt to standardize the feeding was abandoned since variation was not eliminated.

A fourth factor which tended to increase the variation was the fact that the ketone substance production during the control day following administration of sugar was often higher than during the control day preceding the sugar (compare second control day, urine acetone table 2). In a 4-day experiment this would mean that the control values preceding the second sugar would be considerably higher than those preceding the first sugar.

The control periods revealed likewise a diurnal variation which, however, was not perfectly consistent. The majority of dogs showed a higher ketone substance production during the third period (4 to 8 P.M.) than in any other period during the day. From this high level the ketone substance production either decreased (first control period, table 4), or remained

fairly constant during the following period (first control period, table 3). Other controls demonstrate the more ideal conditions, namely a steady increase during the whole day.

Since there is such a wide variation in the degree of ketosis developed in different dogs, the results in the second series of experiments are based on control periods immediately preceding each experiment as indicated in tables 2, 3 and 4 and not on an isolated series of controls, as indicated in table 1.

TABLE 1

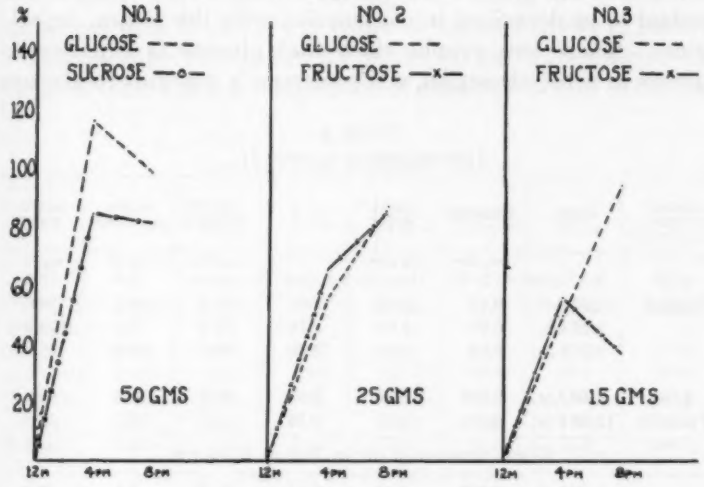
Effect of glucose and sucrose on ketone substance production. Series I

TIME	NITROGEN	URINE SUGAR	D : N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of five controls							
8.00 A.M.	*gm./hr.*	*gm./hr.*		*mg./hr.*	*mg.%*	*mg.%*	*mg./hr.*
12.00 M.	0.418	1.26	3.00	43.7	26.9	41.3	58.2
4.00 P.M.	0.382	1.08	3.09	77.3	36.7	35.8	112.0
8.00 P.M.	0.391	1.04	2.92	95.8	41.7	36.0	113.3
Average effect of 50 gm. glucose given at 12.05 P.M.							
12.00 M.	0.384	1.32	3.44	17.5	22.0	37.5	6.8
4.00 P.M.	0.320	4.20	13.16	11.9	14.2	111.0	— 16.1
8.00 P.M.	0.300	2.18	9.50	4.8	16.0	51.5	1.7
Average effect of 50 gm. sucrose given at 12.05 P.M.							
12.00 M.	0.467	1.46	2.98	22.6	27.2	33.0	37.8
4.00 P.M.	0.328	2.65	8.13	10.2	17.1	94.1	— 16.0
8.00 P.M.	0.315	1.99	6.23	10.8	15.5	60.6	18.0

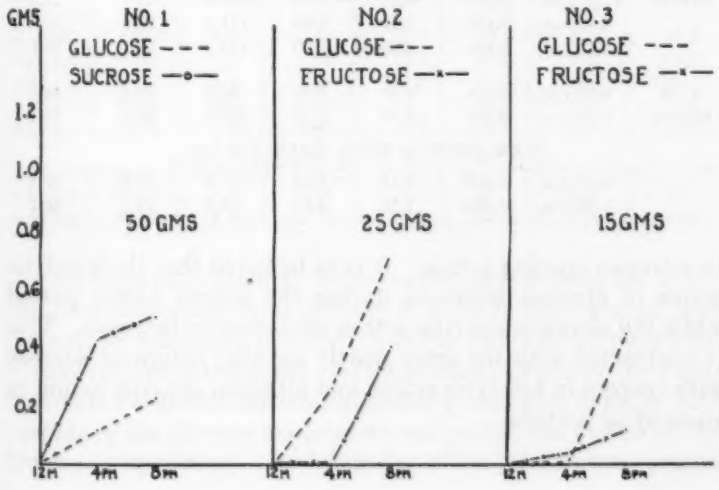
Comparative effect of 50 gm. of glucose and 50 gm. of sucrose

The results of these experiments shown in table 1, make up the first series of experiments to be reported. The average control reported in the table was obtained from a series of five control periods run on three different dogs, only two of which were followed by sugar. The glucose and sucrose experiments represent average figures obtained from three experiments with each sugar and the comparative effect of these two sugars shown in the graph (graph 1, no. 1) is based on the average control periods. The comparative ketolytic effect

% REDUCTION OF KETONE SUBSTANCES
GRAPH 1



TOTAL NITROGEN SPARED
GRAPH 2



shown in graph 1, no. 1, and the comparative nitrogen sparing action shown in graph 2, no. 1, were calculated according to the method to be described in conjunction with the 25 gm. experiments. These two graphs show that glucose is superior to sucrose in ketolytic action, while sucrose is superior to glucose

TABLE 2

Type experiment in series II

DATE 1936	TIME	NITROGEN	URINE SUGAR	D : N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR
		gm./hr.	gm./hr.		mg./hr.	mg.%	mg.%
4/13	8.30 A.M.	....	....	....	....	3.2	56.0
Control	12.25 P.M.	0.81	2.21	2.68	71.0	10.3	24.0
	4.25 P.M.	0.66	2.04	2.72	81.0	9.9	31.0
	8.25 P.M.	0.43	1.69	3.30	59.0	13.8	15.0
4/14	8.40 A.M.	0.856	2.54	2.98	30.6	13.8	37.0
Fructose	12.30 P.M.	0.664	1.43	2.16	77.3	17.7	30.0
15 gm. fructose in 100 cc. H_2O at 12.35 P.M.							
	4.30 P.M.	0.523	2.84	5.44	34.7	3.5	80.8
	8.30 P.M.	0.388	2.09	5.39	8.7	9.6	50.5
4/15	8.45 A.M.	0.851	2.42	2.84	34.4	18.7	43.1
Control	12.45 P.M.	0.616	1.57	2.54	163.0	25.1	28.4
	4.45 P.M.	0.496	1.51	3.04	171.1	25.8	20.8
	8.45 P.M.	0.481	1.43	2.96	199.1	26.7	23.0
4/16	9.00 A.M.	0.854	2.46	2.89	85.6	22.5	44.0
Glucose	1.00 P.M.	0.685	1.59	2.31	219.0	32.2	23.6
15 gm. glucose in 100 cc. H_2O at 1.05 P.M.							
	5.00 P.M.	0.600	3.14	5.24	126.0	20.9	98.1
	9.00 P.M.	0.434	1.98	4.57	29.9	14.5	46.7

in nitrogen sparing action. It is to be noted that the ketolytic action of glucose decreases during the second 4-hour period while the nitrogen sparing action continues to increase. This is contrasted with the more nearly parallel action of sucrose with respect to ketolytic action and nitrogen sparing action in succeeding periods.

Comparative effects of 25 gm. of glucose and 25 gm. of fructose

A typical experiment, two days of which were controls, employed with 25 and 15 gm. doses of sugar is shown in table 2.

The average results for all of the 25 gm. experiments are given in table 3 and a comparison of ketolytic action of the two

TABLE 3

Effect of 25 gm. glucose and fructose

TIME	NITROGEN	URINE SUGAR	D : N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of three experiments with glucose							
	gm./hr.	gm./hr.		mg./hr.	mg.%	mg.%	mg./hr.
8.00 A.M.	....	....	....	....	30.9	36.3	....
12.00 M.	0.569	1.91	3.33	116.4	42.4	31.9	165.3
4.00 P.M.	0.605	1.75	2.90	187.1	46.4	29.7	212.4
8.00 P.M.	0.509	1.54	3.01	180.4	45.3	28.6	182.6
8.00 A.M.	0.81	2.61	3.39	74.9	45.0	55.9	171.3
12.00 M.	0.59	1.77	3.21	131.7	57.5	27.8	204.8
25 gm. glucose in 150 cc. H_2O at 12.05 P.M.							
4.00 P.M.	0.56	4.95	8.95	70.4	47.4	57.7	25.6
8.00 P.M.	0.351	1.70	5.35	12.0	44.3	38.2	8.1
Average three experiments with fructose							
8.00 A.M.	....	....	....	....	45.4	45.5	....
12.00 M.	0.677	2.15	3.33	44.8	49.4	33.2	51.8
4.00 P.M.	0.464	1.57	3.46	98.1	58.2	27.7	134.7
8.00 P.M.	0.455	1.67	3.97	145.2	69.0	28.0	224.5
8.00 A.M.	0.733	2.64	3.70	51.1	47.8	50.7	9.7
12.00 M.	0.617	1.40	2.95	79.0	49.5	33.5	84.6
25 gm. fructose in 150 cc. H_2O at 12.05 P.M.							
4.00 P.M.	0.435	3.62	9.64	32.9	38.3	61.6	— 25.7
8.00 P.M.	0.296	2.32	7.29	9.9	53.3	47.1	76.9

sugars is given in table 4. Ketone substance production previous to administration of glucose is considerably higher than the ketone substance production in the corresponding period of the fructose experiment. After administration of the sugar it is noted that the ketone substance production falls in both periods of the glucose experiment and in but one period of the fructose experiment. Judging the effect of the two sugars

on the basis of reduction in ketone substances from the base level would indicate a decided superiority of glucose over fructose. It is noted, in connection with the controls, however,

TABLE 4

Comparative reduction in ketone substance production by 25 gm. glucose and fructose

CONTROL			EXPERIMENT					
Period	Ketone substance production	Change	Ketone substance production	Assumed level	Reduction	Total production	Total reduction	Per cent reduction
Experiment 1. Glucose [1]								
	mg./hr.	*mg./hr.*	*mg./hr.*	*mg./hr.*	*mg./hr.*	*mg.*	*mg.*	
8–12	20.3		136.7	136.7		544		
12–4	78.7	58.4	— 83.7	195.1	278.8	780	1101	84.0
4–8	28.0	— 50.7	10.4	144.4	133.9	575	535	93.0
Experiment 1. Fructose								
8–12	77.7		174.0	174.0		696		
12–4	96.7	19.0	— 105.0	193.0	289.0	773	1157	81.0
4–8	83.3	— 13.4	12.7	179.6	166.9	719	667	93.0
Experiment 2. Glucose								
8–12	362.5		227.5	227.5		910		
12–4	382.5	20.0	— 1.0	247.5	248.5	989	995	52.0
4–8	359.7	— 22.8	77.9	224.7	146.8	896	587	65.5
Experiment 2. Fructose								
8–12	362.5		164.0	164.0		656		
12–4	382.5	20.0	103.5	184.0	80.5	736	322	23.0
4–8	359.7	— 22.8	27.5	161.2	133.7	644	535	82.5
Experiment 3. Fructose								
8–12	— 25.5		24.7	24.7		99		
12–4	191.6	217.1	— 9.3	241.8	251.1	968	1001	94.5
4–8	581.8	390.2	153.1	632.0	478.9	2530	1915	76.0
Experiment 3. Glucose								
8–12	166.1		306.4	306.4		1225		
12–4	191.6	25.5	127.4	331.9	204.5	1325	819	32.0
4–8	227.2	35.6	11.4	367.5	356.1	1469	1425	97.0

[1] Sugar always given immediately after 12 NOON.

that ketone substance production does not remain at a fixed point, the tendency being a continuous rise throughout the day. It is assumed, then, that ketone substance production

would have risen proportionately the same on each experimental day as it did during the control day. Table 4 was constructed on this assumption, adding to the base level before administration of sugar, the same differences in ketone substance production in successive periods as appeared in the control periods of the previous day. The reduction in ketone substance production, then, is measured from the assumed level, taking the difference between the assumed level and the level as found at each period following administration of sugar. Per cent reduction is calculated from the total amount formed and the total amount reduced, the total amount formed in the period previous to administration of sugar being taken into account in the per cent reduction of the 12 to 4 o'clock period. The average results of the per cent reduction in ketone substance production after administration of 25 gm. of the two sugars are represented in the graph (no. 2, graph 1). From this graph it is seen that the per cent reduction after administration of fructose is slightly greater in the first 4-hour period than after glucose. At the end of 8 hours the average reduction is approximately the same for both sugars. The reduction in ketone substance production affected by fructose falls off more rapidly than the reduction produced by glucose. This, however, is very slight and the significance is questionable.

The nitrogen sparing action, calculated in the same way as the figures in table 4, is graphically shown in graph 2, no. 2. Here it is shown that, although there is a greater ketolytic effect 4 hours after fructose, there is less nitrogen spared in the corresponding period than there is after administration of glucose. The increase in nitrogen sparing action in the second, 4-hour period in each case, coincides with a decrease in the reduction of ketone substance production in the corresponding period.

Comparison of 15 gm. of glucose and 15 gm. of fructose

All of the 15 gm. experiments are averaged in table 5 and the comparative effect of the two sugars on ketone substance

production is shown in detail in table 6. The controls in this series show results which are comparable with those of the previous series and, as before, the base level before the administration of glucose is considerably higher than the base level before administration of fructose. The decrease in ketone substance production following glucose is in the same

TABLE 5

Comparative effects of 15 gm. glucose and fructose

TIME	NITROGEN	URINE SUGAR	D : N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of three experiments with glucose							
	gm./hr.	*gm./hr.*		*mg./hr.*	*mg.%*	*mg.%*	*mg./hr.*
8.00 A.M.	....	....	....	....	27.9	45.8	....
12.00 M.	0.690	1.83	2.64	92.9	27.2	46.8	88.4
4.00 P.M.	0.560	1.71	3.06	125.0	39.7	50.9	178.6
8.00 A.M.	0.510	1.61	3.14	169.0	41.1	24.3	176.0
8.00 A.M.	0.800	2.38	2.97	101.7	68.5	61.2	114.0
12.00 M.	0.630	1.98	3.10	153.0	43.9	29.9	155.9
15 gm. glucose in 100 cc. H_2O at 12.05 P.M.							
4.00 P.M.	0.51	2.98	5.85	100.5	40.5	56.4	88.0
8.00 P.M.	0.34	1.73	4.60	37.0	33.3	43.4	3.9
Average three experiments with fructose							
12.00 M.	0.77	2.09	2.83	125.0	40.9	37.0	130.4
4.00 P.M.	0.62	1.83	3.37	160.0	48.0	43.1	193.9
8.00 P.M.	0.54	1.81	3.41	180.3	56.9	25.1	221.9
8.00 A.M.	0.857	2.43	2.86	147.7	65.7	52.4	191.7
12.00 M.	0.68	1.73	2.51	138.8	41.0	50.3	14.7
15 gm. fructose in 100 cc. H_2O at 12.05 P.M.							
4.00 P.M.	0.52	3.85	7.30	58.8	23.8	74.9	— 15.0
8.00 P.M.	0.42	1.78	4.15	44.1	30.9	58.5	75.1

order as that noted after 25 gm. of the sugar. In both this series and the previous one, it is noticed that 4 hours after fructose there is a negative ketone substance production, possibly due to the fact that the ketone substance production before administration was lower in each case than in the glucose group.

Table 6, constructed in the same way as table 4, shows the comparative effect of the two sugars on ketone substance pro-

duction for all of the 15 gm. experiments. Graphically the per cent reduction in ketone substance production is shown in graph 1, no. 3, and indicates the slight superiority of fructose

TABLE 6

Comparative reduction in ketone substance production by 15 gm. glucose and fructose

CONTROL			EXPERIMENT					
Period	Ketone substance production	Change	Ketone substance production	Assumed level	Reduction	Total production	Total reduction	Per cent reduction
			Experiment 1. Fructose [1]					
	mg./hr.	mg./hr.	mg./hr.	mg./hr.	mg./hr.	mg.	mg.	
8–12	86.6		83.9	83.9		335.6		
12–4	87.7	1.1	— 16.0	85.0	101.0	340.0	404	60
4–8	127.7	40.0	18.8	125.0	106.2	500.0	424.8	85
			Experiment 1. Glucose					
8–12	52.1		35.9	35.9		143.6		
12–4	89.1	37.0	— 14.8	72.9	87.7	291.6	350.8	81
4–8	120.3	31.2	8.1	103.2	95.1	412.8	380.4	92
			Experiment 2. Fructose					
8–12	100.6		93.8	93.8		374.2		
12–4	79.5	— 21.1	— 24.6	71.7	96.3	286.8	385.2	58
4–8	75.2	— 4.3	34.1	67.4	33.3	269.6	134.2	49
			Experiment 2. Glucose					
8–12	190		269.3	269.3		1177.2		
12–4	173.8	— 16.2	79.0	253.1	174.1	1012.4	706.4	33
4–8	202.8	29.0	3.2	282.1	278.9	1128.4	1115.6	98.5
			Experiment 3. Glucose					
8–12	23.3		173.0	173.0		692.0		
12–4	273.0	249.7	202.0	422.0	220.0	1688.0	880	37
4–8	205.0	— 68.0	24.9	354.0	329.1	1416.0	1316.4	93
			Experiment 3. Fructose					
8–12	204		— 101	— 101.0		— 404.0		
12–4	414.5	210.5	— 7.8	99.5	107.0	398.0	430	54
4–8	463.0	48.5	71.8	138.0	+ 33.5	552.0	+ 134	+ 24

[1] Sugar always given immediately after 12 NOON.

over glucose in the first 4-hour period. In the second 4-hour period glucose continues to reduce ketone substance production while fructose drops off markedly.

Nitrogen sparing action, graph 2, no. 3, calculated as before, indicates the superiority of glucose in the second 4-hour period. In contrast to the adjoining graph, neither glucose nor fructose produces any nitrogen sparing action in the first 4-hour period. Evidently the nitrogen sparing property is not positively correlated with ketolysis at this level of feeding the sugars. As the nitrogen sparing action of fructose appears in the second period, the ketolytic effect diminishes, in contrast to the simultaneous increase in nitrogen sparing and ketolytic action of glucose.

DISCUSSION

The conception of ketone substance production is offered as a better measure of ketosis than can be either ketonemia or ketonuria. It assumes obviously that the ketone level of the blood reflects very quickly changes of ketone level in the ketogenic tissues (probably the liver in the main) and that the kidney pretty promptly responds to these changes in level, since no known thresholds for the ketone substances exist. The blood content then represents some rather definite fraction of the ketones present in the whole body, and if only this fraction were known, the term ketone substance production could be corrected factorially.

Disappearance of ketone substance, almost certainly by oxidation, may be measured by the same calculation. Referring to table 4, experiment 1, it is observed that on the first day 780 mg. of ketone substances would have been formed if sugar had not been given. Administration of glucose, however, prevented the formation of that amount and at the same time caused the disappearance of approximately 220 mg. (136.7 + 83.7) of the already-formed 544 mg. This sort of effect is indicated by negative signs in tables 1, 3 and 5. Negative ketone substance production then indicates greater disappearance (oxidation) of blood ketones than the amount excreted through the kidney in the corresponding period.

There are some instances in the tables, however, where demonstrated disappearance of ketones does not show up as a negative production. For example, in table 3, after administration of glucose the average figure does not work out a

negative production, although in table 4 the figures for the first two experiments with glucose clearly indicate that ketones have disappeared. The average ketone substance production before the administration of sugar was sufficiently high to prevent the average reduction from showing as a negative production. Judging by the low positive production at the end of 8 hours, it is probable that a negative production would have appeared in another 4 hours.

As was pointed out by Wierzuchowski ('27) the greatest ketolytic effect follows the largest dose of sugar. Although the ketolytic effect of glucose after 25 and 15 gm. is almost the same, the maximum effect occurs after administration of 50 gm. Also 25 gm. of fructose produce a greater effect than 15 gm. The fact that the ketolytic action following 50 gm. of glucose falls off during the second 4-hour period may be due to complete abolition of the ketosis in the first period since, as seen in table 1, the ketone substance production is very low before the sugar was given.

In the 15 and 25 gm. experiments the nitrogen sparing action of glucose is superior to fructose, and is roughly proportional to the amount of sugar given. The results with 50 gm., however, are contradictory both as to the order of nitrogen sparing and the amount compared with the dose of sugar given. A tentative nitrogen sparing order would be glucose, sucrose and fructose.

That the nitrogen sparing effect of sugars did not turn out to be parallel to or to bear any other definite relationship to ketolytic effect, may be ascribed to inevitable errors of determination or to an inadequate number of experiments, or both. However, this relationship has been rather assumed from the Shaffer-Woodyatt theory of keto-antiketogenesis, than demonstrated by previous investigators. Wierzuchowski ('27) came nearest to a demonstration of such a relationship when he undertook (p. 442) a 'dynamogenic balance' after glucose ingestion in the phlorhizinized dog. He found that the physiological heat value of the protein spared in a series of nine experiments came to a total of 407 Cal. Subtracting from

this the calories represented by 58% of the protein which would have been wasted as sugar, if the protein had been katabolized, there remained 171 Cal. Then calculating the ketolytic effect of the glucose as beta-hydroxybutyric acid and 'supposedly oxidized,' he arrived at the figure 174.4 Cal. He very properly discounted this coincidence for two reasons: 1) the protein moiety remaining after sugar formation almost certainly would have formed some ketone substances, and 2) there was a considerable amount of sugar unrecovered (132.5 Cal.) which must have contributed to the ketolysis, probably, "by direct chemical influence in the sense of Shaffer." A third reason obviously is the improbability that all the ketones were first converted to beta-hydroxybutyric acid before oxidation.

A similar coincidence can be found in the present experiments. Thus the following values may be calculated.

	Protein spared		*Sugar-unrecovered*		*Ketolysis as β-hydroxybutyric acid*	
	gm.	Cal.	gm.	Cal.	gm.	Cal.
6 Exp. Fructose	27.0	118.7	49.1	183.1	12.9	60.7
6 Exp. Glucose	31.4	138.2	47.5	178.0	18.3	85.7

Deducting 58% of the protein lost as sugar there remains 60.4 Cal. spared for fructose and 70.8 Cal. for glucose. One must not be misled by such a striking coincidence as this in the case of fructose, for it is the result of ignoring the several considerations mentioned above. What impresses the present writers in the results tabulated above is the lack of correspondence between the ketolytic effect, expressed either as grams or calories, and the sugar unrecovered in the urine. In a later paper from this laboratory an attempt is made to correlate combustion of carbohydrate with the reduction of ketone substance production brought about by administration of sugar. Meantime it may be added that nitrogen can be spared in phlorhizinized dogs by carbohydrate even though it is not burned, as demonstrated by Gaebler and Murlin ('25). The unburned fraction should also be studied in relation to ketone substance production.

CONCLUSIONS

1. The ketolytic effect of 50 gm. of glucose is greater than 50 gm. of sucrose in phlorhizinized dogs.

2. With small doses of sugar (15 gm.) the ketolytic effect of glucose is greater than fructose. With larger doses (25 gm.) the ketolytic effect of the two sugars is practically the same.

3. A comparison of the nitrogen sparing action and the ketolytic effect of the sugars does not demonstrate any close parallelism between these two functions in the phlorhizinized dog. A tentative nitrogen sparing order is given as glucose, sucrose and fructose.

4. The greatest ketolytic effect follows the largest dose of sugar.

5. The ketolytic effect of sugar is measured by its effect on ketone substance production, a combination of ketonuria and ketonemia, the formula for which is given.

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THE EFFECT OF MONO-, DI- AND TRICALCIUM PHOSPHATES ON REPRODUCTIVE SUCCESS IN RATS

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ONE FIGURE

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We have previously reported the effect of different levels and ratios of calcium and phosphorus on reproductive success in rats (Cox and Imboden, '36). Reference to this earlier report (fig. 1, p. 155) indicates that relatively poor performance can be expected at high levels of mineral intake even when the Ca/P ratio is near to that ordinarily considered optimal (1.0 to 2.0). Because there has been some discussion of the effect of different types of calcium phosphate when fed during reproduction (Maynard, '33; Rottensten and Maynard, '34) we felt that information of some value might be obtained by employing the three calcium phosphates ($Ca_3(PO_4)_2$, $CaHPO_4$ and $Ca(H_2PO_4)_2$) in experiments similar to those previously reported, but at high levels of intake.

EXPERIMENTAL

The basal diet, the technic employed and the method of treating results are described in our previous report (Cox and Imboden, '36). The level of calcium employed was 2.45% of the diet; and, with the exception of the small amount (0.017%) in the basal diet, was furnished by adding each of the three calcium phosphates. At this high level of intake we felt that any differences in the three salts (which might not appear at lower levels) would be manifest.

The experimental mothers were followed for eleven reproductive cycles: the first cycle while receiving stock food, and the remaining ten on the experimental rations. The results are presented in table 1, together with comparable groups from the previous study.

DISCUSSION

We have advanced the thesis (Cox and Imboden, '36) that the optimal ratio of calcium and phosphorus for reproductive success is determined by the calcium level. We found, for example, that at a level of 1.225% calcium, the optimum ratio (on the basis of weight of young at 21 days) was 1.66, and at the 2.45% level, 2.0. No diets with ratios between 1.0 and 2.0, at the highest calcium level, were employed. In the present work results with diets having Ca/P ratios between 1.0 and 2.0, show that the optimum ratio at high levels of calcium intake (2.45%) is about 1.63. This level of calcium, on the basis of the average weight of young at 21 days (46.4 gm.), compared very favorably with any other calcium level, and was superior to the same calcium level (2.45%) at slightly different Ca/P ratio; e.g., Ca/P ratio, 2.0; average weight, 37.3 gm.; Ca/P ratio, 1.15; average weight, 40.0.

At high calcium levels the Ca/P ratios which give young of the largest average weight are almost identical: at 0.735% calcium level, 1.5; at the 1.225% level, 1.66; and at the 2.45% level, 1.63. The interesting fact is that low calcium levels permit a wide range of possible Ca/P dietary ratios with but little deleterious effect on reproductive performance; e.g., four diets at a calcium level of 0.49% and Ca/P ratios between 0.4 and 2.0, gave average weights of young at 21 days of from 41.3 to 46.7 gm. (Cox and Imboden, '36, table 2); whereas at high calcium intakes the possible range of ratio is much narrower (between about 1.7 to 1.2). This fact is illustrated graphically in figure 1. Similar conclusions are also apparent from a consideration of the 'success' ratings.

Because of the wide range of permissible Ca/P ratios when low levels of calcium are used, it is not to be expected that, at

TABLE 1

The effect of a high calcium level on reproduction in rats

DIET					GESTATION				LACTATION			FACTORS EVALUATED			FINAL SUCCESS GRADE
Diet no.	Salts	Per cent Ca	Per cent P	Ca/P ratio	Number completed	Number incomplete	Total living young	Number available to raise	Number completed	Number young raised	Per cent available young raised	Average weight young at 21 days	Ash content grade	Changes in weight mother	
21	.....[1]	2.45	0.245	10.00	2	0	19	12	0	0	0.0	....	.....	— 126	0.5
22	.....[1]	2.45	0.490	5.00	2	0	20	12	0	0	0.0	....	.....	— 40	0.8
23	.....[1]	2.45	0.735	3.33	18	1	124	95	12	57	60.0	19.16	85.7	— 42	20.7
24	.....[1]	2.45	1.225	2.00	21	3	185	119	21	110	92.4	37.33	99.2	+ 31	40.2
33	$Ca_3(PO_4)_2$	2.45	1.50	1.63	40	0	324	216	37	165	76.4	46.37	95.3	+ 64	79.5
32	$CaHPO_4$	2.36[2]	2.06	1.15	46	1	378	251	27	117	46.6	40.01	92.50	+ 71	73.6
25	.....[1]	2.45	2.45	1.00	35	1	295	193	19	82	42.5	33.51	105.10	+ 22	52.8
34	$Ca(H_2PO_4)_2$	2.45	4.01	0.61	5	0	40	30	3	14	46.7	17.20	126.9	— 21	6.0

[1] Consult Cox and Imboden ('36) for composition of salts employed.

[2] Inadvertently. The small difference in level is not considered significant.

such levels, great differences in the two salts, $Ca_3(PO_4)_2$ and $CaHPO_4$ would be observed. Although, on the basis of our earlier work, the latter salt with the lower ratio (1.29) would presumably be preferred to the former (ratio 1.94), at high calcium levels the reverse is true. The 'success' ratings were 79.5 and 73.6, and the average weights of young at 21 days

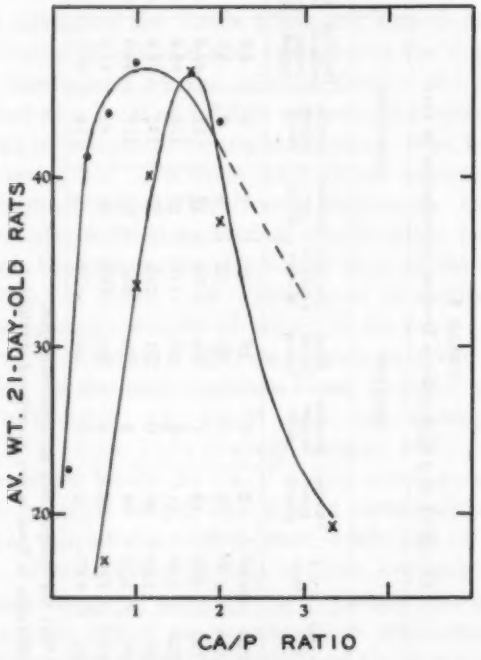

Fig. 1 Average weight of 21-day-old nursling rats from mothers fed diets containing different Ca/P ratios. Diets containing 2.45% calcium are indicated by X; those containing 0.49% calcium are indicated by solid dots.

were 46.4 and 40.0 gm. for $Ca_3(PO_4)_2$ and $CaHPO_4$, respectively. From comparisons on a surface area basis it was suggested (Cox and Imboden, '36) that a calcium level of 0.245% was equivalent to a human calcium intake of 1.37 gm. daily during gestation; the present level (2.45%) would therefore be equal to a daily human intake of 13.7 gm. calcium, as

the food intake of the two groups of rats was strictly comparable.

Maynard ('33) in his review on the value of calcium supplements in animal feeding could find no preponderance of evidence in favor of any one form of calcium. In his further work with Rottensten and Maynard ('34) on the effect of different calcium phosphates on bone ash and growth in rats, he could reach no definite conclusion regarding the superiority of the secondary or tertiary phosphate. The femurs of our mother rats showed 61.92, 62.22 and 61.79% bone ash for the primary, secondary and tertiary phosphates, respectively.

From table 1 it is evident that increase in dietary phosphorus resulted in progressively higher ash grades, even when the Ca/P ratio became less than 1.0. This has been discussed in our previous report, and it was pointed out that excessively high body ash content when due to an excess of phosphorus is not indicative of correspondingly high bone ash.

We consider it of additional interest to note that even at the high calcium level employed and the absence of vitamin D, the mother rats on the secondary and tertiary phosphates showed no gross signs of excessive mineral intake—either as noted during life, or at autopsy.

SUMMARY

Experimental rats were observed for eleven reproductive cycles to determine the effect of primary, secondary and tertiary calcium phosphates fed at a high calcium level. At a level of 2.45%, the range of Ca/P ratio which allows optimum reproductive performance is much narrower than when low calcium levels are employed, and is between 1.7 and 1.2.

At this calcium level (2.45%) the primary calcium phosphate is definitely unsuitable for reproductive success. The tertiary phosphate is slightly superior to the secondary, but this difference would probably not be apparent at lower calcium levels.

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THE RESPONSE OF RATS, CHICKS AND TURKEY POULTS TO CRYSTALLINE VITAMIN G (FLAVIN)

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SIX FIGURES

(Received for publication June 26, 1936)

Goldberger and Lillie ('26) demonstrated the presence of two factors in the vitamin B complex by feeding to rats an 85% alcoholic extract of white corn as a source of vitamin B. Growth was arrested, and the animals developed a dermatitis which the authors termed 'pellagra-like.' The syndrome was cured by autoclaved yeast, or by a fuller's earth adsorbate prepared from an aqueous extract of autoclaved yeast. The curative factor subsequently became known as vitamin G or B_2. Kuhn, György and Wagner-Jauregg ('33) finally crystallized vitamin G (flavin) with the aid of the adsorption technic. György ('34) soon afterward showed clearly that purified vitamin B and vitamin G (flavin) are incapable of fully satisfying the rats' requirements for the vitamin B complex. He maintained that a third factor, vitamin B_6, was also necessary. Later work on the vitamin B complex has shown that the question is an involved one; Elvehjem and Koehn ('35), Birch et al. ('35), Koehn and Elvehjem ('36), Dann ('36), Elvehjem and Koehn ('36).

It was thought advisable to reinvestigate vitamin G (flavin) with the precautions now available to guard against a deficiency of other factors. By the addition of crystalline vitamin G (Lepkovsky et al., '35) it was possible to restore to normality rats rendered deficient by its absence. This served as a suitable criterion for the adequacy of the basal diet with respect to other factors.

EXPERIMENTAL

Diets used. The composition of the diets is shown in table 1. Diet 2 was supplemented daily with 2 drops of cod liver oil,[1] 6 units of a highly potent vitamin B concentrate,[2] and 0.5 cc. of rice bran filtrate 2[3] (Lepkovsky and Jukes, '35). The lard, cornstarch, yellow corn meal, wheat middlings, ground limestone, iodized salt and bone meal were ordinary commercial products. Salt mixture 2 consisted of bone ash, 38%; calcium

TABLE 1

Composition of the diets used

	DIET 2 (RATS)	DIET 92 (CHICKS)	DIET 90 (TURKEYS)	DIET 60 (TURKEYS)	DIET 70 (CHICKS)[a]
Casein, acid washed	27 gm.	20	22	18	
Sucrose	59				
Lard	10				
Salt mixture 185[b]	4				
Salt mixture 2		3	3		
Cornstarch		50	36		
Wheat bran, acid washed		20	20		
Yellow corn meal				53	
Wheat middlings				22	
Liver filtrate		4 cc.		4 cc.	
Rice bran filtrate 1			15 cc.		
Ground limestone				1	
Calcium carbonate, precipitated			1		
Sodium chloride, iodized				1	
Bonemeal				1	
Cod liver oil, medicinal	*	2	3	2	
Hexane extract of alfalfa meal, 1 cc. ≏ 2 gm.		0.5 cc.	0.5 cc.	0.5 cc.	
Tap water		4			
Vitamin B concentrate 1[c]		0.3 cc.			
Vitamin B concentrate 2[d]			0.1 cc.		

[a] Lepkovsky and Jukes ('35).
[b] McCollum and Simmonds ('18).
[c] Assayed with chicks, and fed at a level to provide a 50% excess.
[d] Assayed with chicks, and fed at three times the level necessary for chicks.
[e] See below.

[1] The cod liver oil was generously presented by Mead Johnson and Co., Evansville, Indiana.

[2] The vitamin B was kindly furnished by Dr. Elmer Stuart of the Lilly Research Laboratories, Indianapolis, Ind.

[3] The Vitab Products, Inc., San Francisco, furnished the rice bran concentrates, the fullers' earth adsorbate, and the vitamin G concentrates.

carbonate, 20.5; iodized salt, 23; magnesium sulfate, 7.5; potassium dihydrogen phosphate, 7.5; ferric citrate, 3.5. Wheat bran was washed twice by standing overnight in several volumes of dilute sulfuric acid at a pH of about 3, and dried at 60° to 70°. Liver filtrate and rice bran filtrate 1 have been previously described, except that the liver filtrate was treated twice with fullers' earth.[4] The filtrates were assayed with chicks (Lepkovsky and Jukes, '36), and fed at a level supplying at least twice the necessary amount of the 'filtrate factor' for maximal growth on the heated diet.

The alfalfa extract was prepared by Dr. H. J. Almquist by extracting alfalfa meal with hexane in a continuous extractor, and assayed by him for the anti-hemorrhagic vitamin. The level fed supplied at least twice the amount necessary to protect against hemorrhages on his basal diet (Almquist, '36).

Vitamin B concentrates 1 and 2 were prepared from a fullers' earth adsorbate[5] (McCollum and Simmonds, '18) of an aqueous extract of rice bran by elution with baryta, which does not dissolve vitamin G (flavin).

Crystalline vitamin G was prepared from a highly potent vitamin G concentrate[5] by fractional precipitation from water, and recrystallizing the active fractions from hot water.

Care of animals. Rats were kept on wire screen floors. They were fed on basal diet 2 for 10 days, and then the vitamin B and rice bran filtrate were added. The rats were considered ready for use after they ceased to gain in weight.

The care of chicks has been previously described (Lepkovsky and Jukes, '35). Feed consumption records were kept.

Bronze turkey poults were housed in electrically heated battery brooders with wire floors and fed the stock diet (Lepkovsky and Jukes, '35) for 12 days following hatching. They were then placed on basal diets 60 and 90 for 3 days when they were weighed and divided into two groups on each diet, one group receiving vitamin G in addition to the basal diet.

[4] The fullers' earth was purchased from Eimer and Amend, New York.

[5] See footnote 3, page 516.

Results with rats. On the low vitamin G diets rats grew very little or not at all. Their fur became quite generally greasy. Loss of hair occurred in greater or less degree in many rats on the head, neck, shoulders, back and flanks; sometimes small yellow scales appeared on the skin. The paws and face were generally bloody, and sometimes the bloodiness extended over the entire fur. Dried bloody or serous encrustations on the borders of the ears were very characteristic. The eyelids were generally red and swollen, and were sometimes stuck shut. Often the vibrissae were encrusted with dried blood. The feet were generally dirty with dried yellowish secretions. The tail was often dirty and sticky. One of the most characteristic symptoms resulted from gastro-intestinal disturbance causing the rat's abdomen to become distended, and diarrhea sooner or later to appear.

If vitamin G was not fed at this point, the rats slowly lost weight, the diarrhea became more severe, and death followed. Sometimes rats slowly gained in weight without, however, the symptoms entirely disappearing. On close observation, such rats generally revealed coprophagy.

Addition of 10 to 20 micrograms of vitamin G daily to the diet caused an immediate and marked response in the rats. Figure 1 shows the course of improvement of a rat near death from lack of vitamin G. At the time it was first photographed (fig. 1, a) it was in a miserable and greasy condition, it was badly denuded, covered with small yellow scales, its ears and vibrissae were encrusted with dried secretions, and one eye was stuck shut. The addition of 10 micrograms daily of vitamin G for 5 days brought about a spectacular change (fig. 1, b) and the rat, while not normal in appearance, was greatly improved, and gained 13 gm. After 20 days (fig. 1, c), the rat was normal in appearance and had gained 21 gm. in weight. The rat seemed permanently injured since it gained weight but very slowly.

Ordinarily, diarrhea appeared irregularly in the vitamin G deficient rats. Diarrhea developed rather suddenly in thirteen of a group of seventeen rats; four developed a very severe

diarrhea, and nine were milder cases. All seventeen rats were then given 20 micrograms of vitamin G daily. One of the four rats with severe diarrhea died, but the other three responded immediately, and in nineteen days gained an average

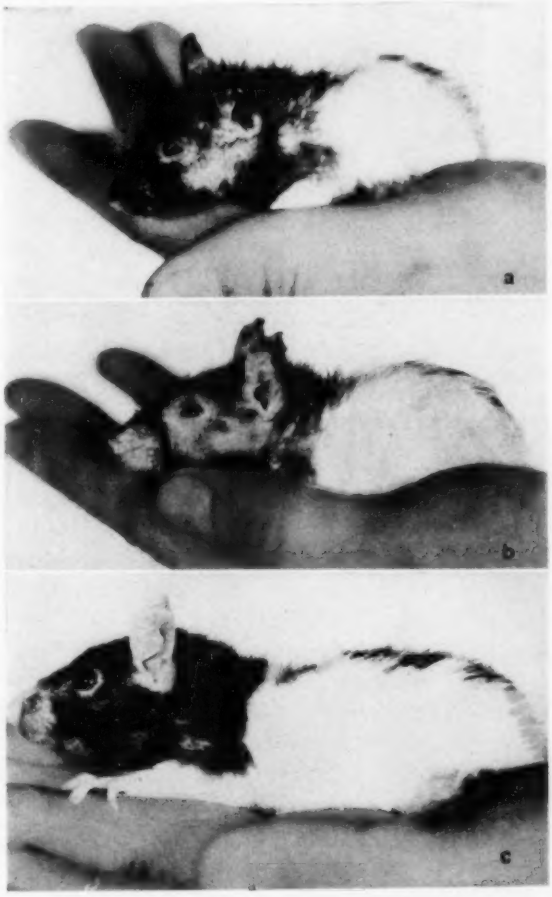

Fig. 1 Illustrating the effect of crystalline vitamin G (lactoflavin) upon a rat with acute vitamin G deficiency; a, before supplementation; b, after 5 days of feeding a daily supplement of 10 micrograms of vitamin G (lactoflavin); c, after 20 days of supplementation.

of 53 gm. in weight. While the diarrhea decreased, it did not entirely disappear, and kept recurring in a milder form. The nine rats with mild diarrhea lost the diarrhea completely

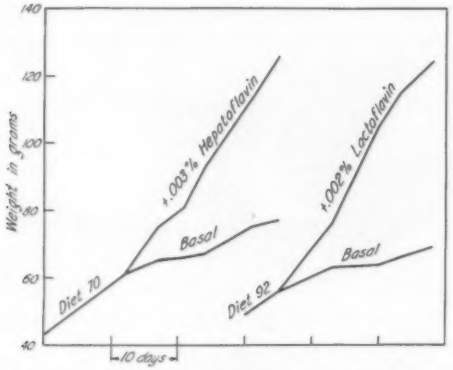

Fig. 2 Growth of chicks with and without crystalline vitamin G (flavin). Ten chicks were used in each of the four experimental groups.

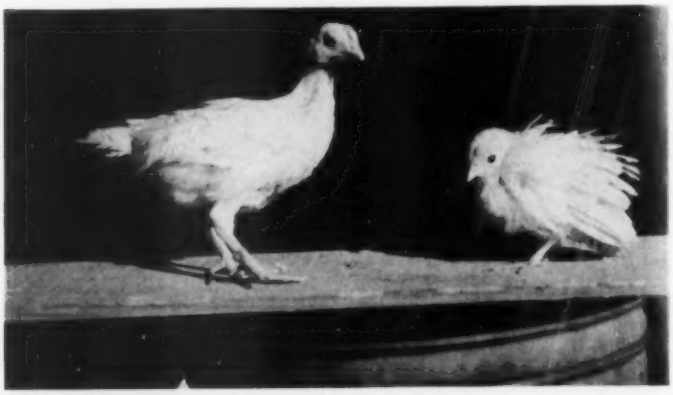

Fig. 3 Thirty-four-day-old chicks, one at right on basal diet 92, one at left on diet 92 plus 2 mg. of crystalline vitamin G (lactoflavin) per 100 gm. of diet.

and gained in 19 days an average of 46 gm. Those without diarrhea gained an average of 40 gm. in 19 days. The response in weight seemed to be in inverse ratio to the severity of the diarrhea. Other symptoms disappeared.

Fig. 4 Twenty-nine-day-old turkey after 17 days on flavin-deficient diet 60. Note the stuck eyelids and the encrustation of the mouth and nostrils.

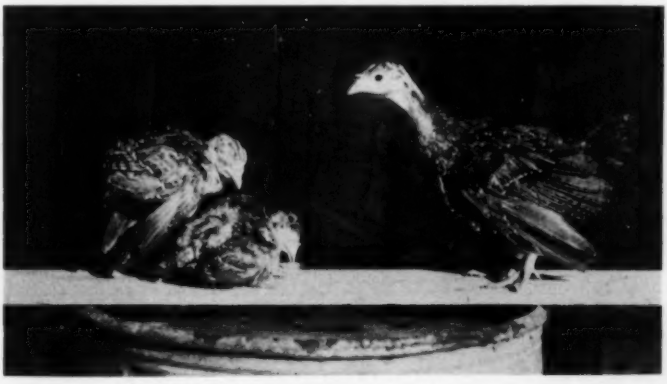

Fig. 5 Twenty-nine-day-old turkeys; two at left after 17 days on basal diet 60, one at right on diet 60 supplemented with 2 mg. of crystalline vitamin G (lactoflavin) per 100 gm. of diet.

Results with chicks. Chicks on basal diet 92 (table 1) or basal diet 70 (Jukes and Lepkovsky, '35) grew very slowly and became weak and emaciated, but retained a fairly good appetite. Diarrhea developed after 8 or 10 days. There were no symptoms of dermatitis. Mortality was high after about 3 weeks. Growth of feathers did not seem to be impaired, indeed, the main wing feathers appeared to be disproportionately long in comparison with the body. The addition of vita-

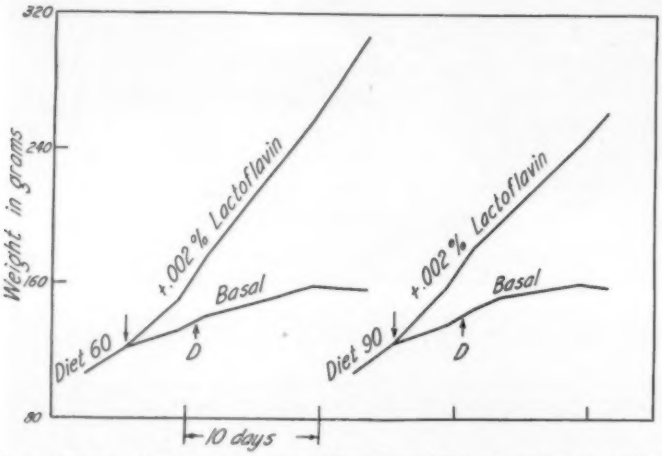

Fig. 6 Growth of turkeys with and without crystalline vitamin G (lactoflavin). Ten or eleven turkeys were used in each of the four experimental groups. 'D' indicates the appearance of dermatitis.

min G caused a marked response in growth, and in the efficiency with which food was utilized for growth. The birds receiving vitamin G appeared fairly healthy, but they did not grow so fast as chicks on a complete 'natural' diet. They did not develop diarrhea. Figures 2 and 3 illustrate the effect of the addition of vitamin G.

Results with turkeys. Dermatitis started to appear in turkeys after 8 days on either diet 60, consisting of natural foodstuffs, or diet 90, which was a more purified diet. The external symptoms, illustrated in figures 4 and 5, were similar

to those of chick dermatitis as first described by Ringrose, Norris and Heuser ('31), but the vent also became encrusted, inflamed and excoriated. Growth slowed up and completely ceased by the seventeenth day. Deaths started to occur on the twenty-first day. Supplementation with 2 mg. of vitamin G (lactoflavin) per 100 gm. of diet prevented the appearance of these symptoms during the experimental period of 3 weeks. Growth was rapid, and there was no mortality. The effect of vitamin G is illustrated in figures 5 and 6. Records of the feed consumption showed, as in the case of the chicks, a greater efficiency in the feed utilization. The birds on basal diet 60 consumed 7.0 gm. of feed per 100 gm. of live weight, and gained 0.20 gm. per gram of feed consumed. With the addition of vitamin G, 9.4 gm. of feed were consumed per 100 gm. of live weight, but the gain per gram of feed was increased to 0.53 gm. Similar results were obtained with diet 90.

DISCUSSION

There are good grounds to believe that many of the previous investigations of vitamin G deficiency were concerned with a multiple deficiency (Lepkovsky, Jukes and Krause, '36). It is thought that the experiments reported in the present communication deal more nearly with a single deficiency, since the high potency of the crystalline vitamin G (lactoflavin) employed indicates that it closely approximated to purity. Vitamin G (lactoflavin) restored rats to normal as far as could be judged by the short experimental periods employed. The symptoms developing in the deficient rats were more or less uniform. Coprophagy was a disturbing factor and tended to obscure the results. It seems clear that a dermatitis of a definite type appeared which could be cured with vitamin G. It is distinctly different from the dermatitis described by György ('35) for 'vitamin B_6' deficiency. The dermatitis had much in common with that described by Goldberger and Lillie ('26). There were, however, some striking differences. The ears, while covered with a "yellowish incrustation of dried serum," were not 'reddened' nor 'thickened' nor, 'in healing'

did it leave "the skin of the pinna with a polished, glistening, and somewhat parchmentlike appearance." No "linear fissuring or ulceration at the angles of the mouth" were noticed. Other observations in this laboratory indicate Goldberger and Lillie were not working with a single deficiency (Lepkovsky, Jukes and Krause, '36). In contrast to these findings are those of Dimick, Smith and Davis ('36) who found no skin symptoms in rats nor any appreciable diarrhea. Dimick and co-workers, however, used different basal diets and vitamin B supplements from those reported in this investigation.

The contrast in symptoms between chicks and turkeys is very striking in view of the fact that these species belong to the same family of birds (Evans, 1899). Furthermore, the dermatitis developed by turkeys deficient in vitamin G is similar to the dermatitis developed by chicks suffering from a deficiency of an entirely different factor (Lepkovsky and Jukes, '35). This factor (the 'filtrate factor') was included at a high level in basal diets 92, 90 and 60. If turkeys are used as a criterion, vitamin G is the 'anti-dermatitis vitamin,' but if chicks are being studied, the title of 'anti-dermatitis vitamin' belongs to the filtrate-factor (Lepkovsky and Jukes, '36). This serves to caution against the too ready identification of dermatitis with specific deficiencies.

The purified diets 92 and 90 were not complete but the observations made over the period of study, with and without vitamin G, probably represent observations definitely related to this vitamin.

SUMMARY

1. Diets deficient in vitamin G (flavin), but which supplied adequate amounts of the other members of the vitamin B complex, were devised for rats, chicks and turkey poults. Vitamin G deficiency was studied by means of these diets, which permitted approximately normal growth and appearance when supplemented with small amounts of crystalline vitamin G (flavin).

2. Dermatitis and other symptoms associated with vitamin G deficiency in rats are described.

3. The symptoms of vitamin G deficiency in chicks were slow growth, diarrhea and emaciation, without dermatitis.

4. An acute dermatitis developed in turkey poults due to vitamin G deficiency, in spite of the fact that the diet was amply supplied with the 'filtrate factor,' which prevents dermatitis in the chick (Lepkovsky and Jukes, '36). The dermatitis was completely prevented by vitamin G (lactoflavin).

5. Vitamin G deficiency in chicks and turkeys appeared to reduce the efficiency of utilization of food more conspicuously than the appetite.

6. The results are discussed briefly in relation to previous investigations of the vitamin B complex.

The assistance of K. K. Miya in the care of the chicks and turkeys is gratefully acknowledged.

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THE ANTIRACHITIC VALUE OF IRRADIATED YEAST IN INFANTS

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ONE PLATE (FOUR FIGURES)

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The earliest report on the efficacy of irradiated yeast as a source of vitamin D for humans was made by Hess ('27). He found that the daily administration of 0.5 to 1.0 gm. of irradiated yeast to rachitic infants brought about a definite curative process. The vitamin D potency of the yeast was not mentioned. In 1930, Kon and Mayzner found that the daily administration of 0.75 gm. of irradiated yeast to rachitic infants brought about a complete disappearance of the symptoms in the course of 6 to 8 weeks, with a rapid rise in the inorganic blood phosphorus and x-ray evidence of advanced healing. In a subsequent article Kon ('31) reported that the yeast used contained 1 Steenbock unit of vitamin D in 1 mg. This means that the 0.75 gm. of the irradiated yeast which was administered daily to the rachitic infants contained 2025 International vitamin D units. Compere, Porter and Roberts in 1935 reported the curative effect on rachitic infants of the daily administration of 6755 International vitamin D units in the form of irradiated yeast.

During the past 3 years we have found that comparatively small amounts of vitamin D in various forms prevented the development of rickets in infants (Drake, '36). The present paper is a report on the effect of the daily administration of approximately 500 International vitamin D units as irradiated

yeast in the prevention and cure of rickets. For ease of administration the irradiated yeast was mixed with farina.[1] The administration twice daily of 2 heaping tablespoons of the cooked farina containing the irradiated yeast furnished 500 International vitamin D units.

PREVENTIVE TESTS

For the prevention of rickets sixty-nine infants were observed over a period of 5 winter months. They represented a cross section of the infant population of Toronto and ranged in age from 4 to 7 months at the initial examination in October and November (table 1). The infants were examined clinically and by x-ray for the presence or absence of rickets according to the procedure as previously reported Drake, Tisdall and

TABLE 1

Ages of infants in months at time of initial examination

GROUP	UNDER 1 MONTH OF AGE	1 MONTH OF AGE	2 MONTHS OF AGE	3 MONTHS OF AGE	4 MONTHS OF AGE	5 MONTHS OF AGE	6 MONTHS OF AGE	7 MONTHS OF AGE	TOTAL
Irradiated yeast	0	0	0	0	13	27	23	6	69
No vitamin D	1	12	19	18	16	7	2	0	75

Brown ('34). Almost all gained in weight at or above the expected rate. In addition to cow's milk dilutions they received rusks and vegetable soup at 7 to 9 months of age. Practically none received egg yolk. Care was taken to see that no vitamin D was obtained from any source other than the irradiated yeast. We were also able to observe a series of infants receiving no vitamin D over the same period.

We are again indebted to Dr. Martha Eliot, assistant chief, Children's Bureau, Department of Labor, Washington, who kindly read the x-ray plates for us. The degree of rickets was classified by Doctor Eliot as 1–, 1, 2 and 3. In this paper we have called Doctor Eliot's group 1–, extremely slight rickets

[1] We are indebted to the Quaker Oats Company for supplying us with the farina containing the irradiated yeast.

(a barely perceptible change which frequently might not be noted); group 1, mild rickets (a slight but obvious rachitic change); groups 2 and 3, moderate and marked rickets (well-defined fringing and cupping at the end of the bone). In addition, the x-ray films were interpreted by Doctor Eliot as to the presence or absence of active advancement of the rachitic process. It should be noted that this last classification indicates only the state of activity of the rachitic process and does not indicate in any way the degree of rickets.

Although we have considered these infants from the standpoint of the prevention of rickets, extremely slight or mild rickets was present in some when the vitamin D therapy was instituted in the fall. Before the initial examination in the fall no vitamin D had been given by mouth although naturally

TABLE 2

Degree of rickets at the initial examination in autumn

GROUP	TOTAL CASES	NO RICKETS	EXTREMELY SLIGHT RICKETS	MILD RICKETS	MODERATE AND MARKED RICKETS
		%	%	%	%
Irradiated yeast	69	81	16	3	0
No vitamin D	75	88	11	1	0

most of the infants had received the vitamin D benefit of summer sunshine. In Toronto very little vitamin D effect of sunshine is obtained after the middle or end of September. In table 2 is given the degree of rickets which was found in the infants in the two groups at the initial examination. It is seen that the number of cases showing evidences of extremely slight and mild rickets is slightly higher in the irradiated yeast group.

In table 3 is shown the maximum degree of rickets observed at any time subsequent to the initial examination.

In table 4 is given the number of cases in each group showing actively advancing rickets with no evidence of healing at times of observation. As stated before, it should be noted that this classification indicates only the presence of activity in the rachitic process and does not indicate in any way the degree of rickets.

CURATIVE TESTS

In order to determine the curative effect of irradiated yeast we gave one infant with marked rickets approximately 1000 International vitamin D units daily in the form of the cooked irradiated yeast—farina mixture. This infant was 7 months of age when the treatment was started on March 15, 1936. A comparison of figure 1, taken March 15th, with figure 2, taken April 15th, shows the curative effect as evidenced by x-ray. Although craniotabes cannot be taken as evidence of the presence or absence of rickets (Drake, Tisdall and Brown, '34), it is of interest that in this case on March 15th an exceedingly

TABLE 3

Maximum degree of rickets observed at any time subsequent to initial examination

GROUP	TOTAL CASES	NO RICKETS	EXTREMELY SLIGHT RICKETS	MILD RICKETS	MODERATE AND MARKED RICKETS
		%	%	%	%
Irradiated yeast	69	87	4	9	0
No vitamin D	75	51	16	20	13

TABLE 4

Number of cases showing actively advancing rickets with no evidence of healing at the times of observation

GROUP	TOTAL CASES	AUTUMN	MIDDLE OF WINTER	END OF WINTER
		%	%	%
Irradiated yeast	69	6	4	0
No vitamin D	75	9	21	31

marked degree of craniotabes was present, while 1 month later the craniotabes had entirely disappeared.

To four other infants with marked rickets we gave approximately 500 vitamin D units daily in the form of the cooked irradiated yeast—farina mixture. In every instance marked healing resulted, as shown by x-rays taken 1 month later (figs. 3 and 4). The x-rays showing evidences of healing were taken on February 1st, April 18th, April 24th and May 14th. The weather in Toronto during the month of April 1936 was so inclement that there was no possibility of the infants receiving any antirachitic effect from exposure to sunshine or

skyshine. (Dominion Meteorological Department reports that in Toronto the month of April 1936 was the darkest April on record.)

DISCUSSION

The results reported in table 3 show that no infant receiving 500 International vitamin D units developed moderate or marked rickets, while 13% of the infants receiving no vitamin D developed rickets of this degree. In table 4 we see that 31% of the infants receiving no vitamin D exhibited active rickets at the concluding observation in March or April, while none of the group receiving the irradiated yeast showed any evidence of actively advancing rickets. It should be noted that the infants in the group receiving no vitamin D are somewhat younger than the infants receiving the irradiated yeast. It is recognized that these younger infants are somewhat more susceptible to rickets.

It is evident from figures 1, 2, 3 and 4 that the daily administration of approximately 1000 or 500 International vitamin D units in the form of irradiated yeast not only prevented the development of rickets but brought about in 1 month's time marked healing of severe rachitic processes.

SUMMARY

1. The daily administration for 5 winter months of approximately 500 International vitamin D units in the form of irradiated yeast to sixty-nine normally growing infants of British and Northern European stock prevented the development in every instance of rickets of a moderate or marked degree.

2. The daily administration of approximately 1000 International vitamin D units in the form of irradiated yeast to one infant with marked rickets and of approximately 500 units to four infants with moderate or marked rickets brought about definite healing in the course of 1 month.

LITERATURE CITED

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PLATE 1

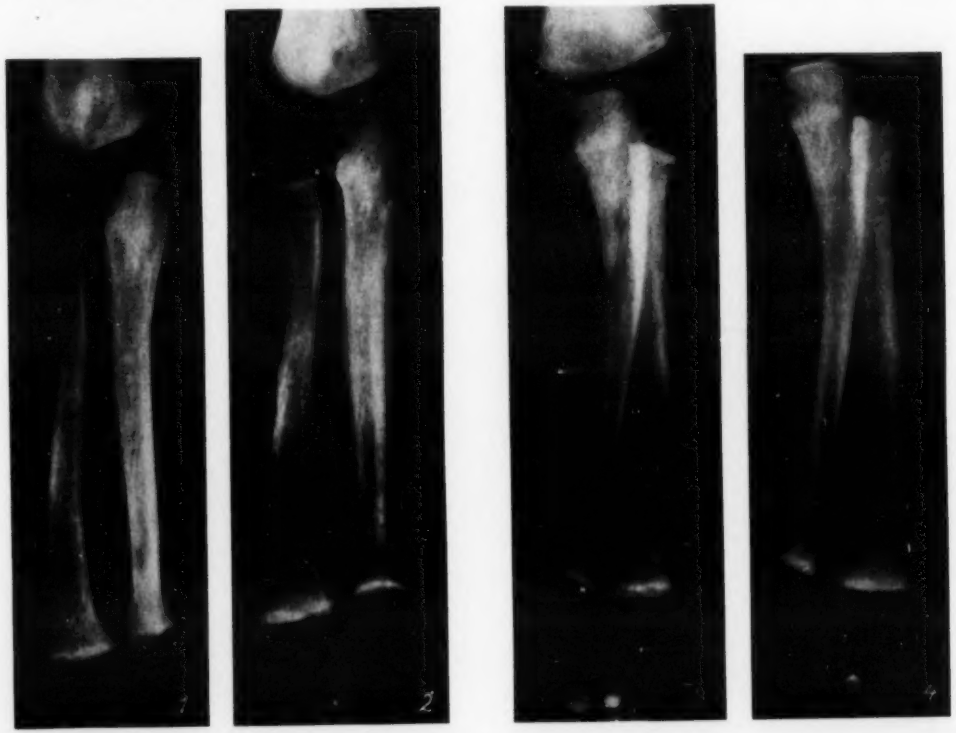
EXPLANATION OF FIGURES

1 Case no. 40. X-ray of wrist of 7-month-old infant with marked rickets, March 15th. This x-ray shows evidences of marked rickets.

2 Case no. 40. X-ray of wrist of the same child, April 15th, after the daily administration for 1 month of 1000 International vitamin D units of irradiated yeast.

3 Case no. 224. X-ray of wrist of infant aged 7 months showing evidences of marked rickets, April 14th.

4 Case no. 224. X-ray of wrist of same infant after the daily administration for 1 month of 500 International vitamin D units of irradiated yeast.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